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A CYTOLOGICAL STUDY OF HETEROTHALLISM IN PUCCINIA GRAMINIS[1]

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INTRODUCTION

Among the lower plants there are species with sexual reproduction in which the two cells that fuse may be borne on the same gametophyte plant. These are spoken of as homothallic. In others the two cells must be borne on separate plants of different sex or strain. In many cases there is no visible difference between the two, and on morphological grounds they can not be classified as male or female. There exist, none the less, well-defined chemical or physiological or genetic differences, such that two reproductive cells of the same plant, or two of different plants of the same sex, can not fuse. Only when two of different strains or sexes are brought together does sexual reproduction take place. Such species are heterothallic.

Data concerning heterothallism in the fungi have been accumulating rapidly in recent years. It is now known to exist in certain species of the mucors, oomycetes, ascomycetes, basidiomycetes, smuts, and rusts.

Certain species of the mucors produced zygospores rarely and under little-understood conditions. Blakeslee (*5*)[3] in 1904 found that by bringing together strains of different origin and growing them side by side, in certain cases a rich growth of zygospores would appear along the line where the two mycelia met. Further experiments showed that all of the geographic strains of a given species that were collected could be classified into two groups. Those of one group would not reproduce sexually with other members of the same group but would with any member of the other group. The two groups were morphologically alike save for a slight difference in vegetative vigor. The more vigorous was called (+), the other (−). In homothallic species, on the other hand, a single-spore culture would produce zygospores.

In this and later papers (*5, 6, 8, 9, 10, 11*) Blakeslee and his associates found that a (+) race of one heterothallic species when grown

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[3] Reference is made by number (italic) to "Literature cited," p. 611.

in contact with a (−) race of another species would undergo the initial stages of sexual reproduction. This so-called "imperfect hybridization" proved of value in deciding which strains were (+) and which (−), as new species came under observation. Moreover, it was found that a homothallic species would show imperfect hybridization with both (+) and (−) strains of heterothallic species. The mycelium of homothallic species is bisexual, that of heterothallic species is unisexual.

The genus Parasitella, which lives parasitically on other molds, was studied by Burgeff in 1924 (*13*) and by Satina and Blakeslee in 1926 (*49*). In *Parasitella simplex* Bainier, living on *Absidia glauca* Hagen, the relation is sex-limited. At the point of attack the relation of host and parasite closely resemble imperfect hybridization, and a (+) strain of the parasite lives only on a (−) strain of its host, and a (−) strain on a (+) of the host. On Rhizopus, on the contrary, the same strain of Parasitella can live on both (+) and (−) strains of the host.

Satina and Blakeslee (*48, 50, 51, 52, 53*) carried on a series of studies of the biochemical differences between (+) and (−) sexes in mucors. The Manoilov reaction gave 85 per cent correct determination of sex in mucors, the (+) being female and the (−) male. Several significant quantitative differences were found between (+) and (−) strains, including their ability to reduce tellurium salts and potassium permanganate.

Couch (*17*) in 1926 reported the existence of heterothallism in Dictyuchus, a water mold of the oomycetes. Several lots of material collected bore sporangia only and were later tested in crosses and proved to be either male or female. Others showed sexual reproduction, and from each of these both male and female strains were isolated. No morphological difference between isolated male and female plants was noted. Only when opposite strains were grown in contact did sexual organs form. Some strains remained neutral. One parthenogenetic strain was found which bore oogonia and eggs without antheridia. It was probably bisexual, however, for when grown with a female strain it developed antheridia, and when grown with one of the male strains the latter developed antheridia.

In the ascomycetes, Derx (*21*) found heterothallism in *Penicillium luteum* Zuk. Monosporous cultures differed macroscopically and chemically. They bore no fertile perithecia, although a few haploid structures without spores were formed. Two by two in certain combinations, these monosporous cultures produced normal perithecia.

Dodge (*26, 27, 28*) found an interesting situation in the ascomycete Neurospora. *Neurospora tetrasperma* Shear and Dodge produced normally in each ascus four binucleate, bisexual spores which grew and reproduced homothallically. Occasionally a smaller uninucleate, unisexual ascospore formed, which grew into a unisexual plant that could reproduce only heterothallically. *N. tetrasperma* also produced asexually a considerable percentage of unisexual conidia. Another species, *N. sitophila* (Mont.) Shear and Dodge, normally heterothallic, produced in each ascus eight uninucleate, unisexual spores. When a unisexual spore of *N. tetrasperma* was grown with a spore of *N. sitophila*, hybrid perithecia resulted. Second generation hybrids have been grown. Wilcox (*61*) found that in *N. sitophila*

the segregation of sex factors took place in the second nuclear division in the ascus.

Bensaude (*2*) in 1918 found heterothallism in *Coprinus fimetarius* L. The primary mycelium from the basidiospore was gametophytic in character. It produced oidia in abundance. An isolated monosporic mycelium remained gametophytic indefinitely, but when a (+) and a (−) mycelium were grown together, fusion took place between mycelial cells or between an oidium of one and a mycelial cell of the other, and at once sporophytic mycelium appeared with binucleate cells showing the characteristic clamp connections.

Since then a voluminous literature has grown up concerning heterothallism in the basidiomycetes. It deals predominantly with the genus Coprinus, which contains both heterothallic and homothallic species.

Typical of these papers is one by Hanna (*32*) on *Coprinus lagopus* Fr. Isolated monosporous mycelia remained gametophytic and without clamp connections. They sometimes produced imperfect haploid fruit bodies, a few of which formed spores which were all of the same sex as that of the mycelium bearing them. When monosporous mycelia from the same fruit body were paired in all possible matings, certain of the matings showed clamp connections (indicative of the sporophytic generation) and others did not. The results showed "that the spores of a single fruit body of *Coprinus lagopus*, while alike morphologically, may be divided sexually into four distinct groups regardless of where the fruit body producing the spores is obtained" (*32, p. 439*). The four spores from a single basidium were sometimes of two sexual groups and sometimes of four. When, however, monosporous mycelia of one geographic strain were mated with those of another strain, there was complete fertility, i. e., 100 per cent of the matings produced clamp connections, thus showing that the sexual groups in all of these fruit bodies must be different. "In the 6 fruit bodies studied * * * there have been established therefore, not 4, but 24 distinct sexual groups" (*32, p. 440*).

The same condition of complete interfertility, or something approximating it, of geographic races of a species has been found by Kniep (*37*) in *Schizophyllum commune* Fr.; by Vandendries (*58, 59*) in *Panaeolus campanulatus* (L. and Fr.) Quelet. and *Coprinus radians* (Desm.) Fr.; by Newton (*41*) in *C. rostrupianus* Hansen; by Brunswik (*12*) in *C. friesii* Quelet., *C. comatus* Battarra, *C. fimetarius* (L.) Fr.; and by Mounce (*39*) in *Fomes pinicola* (Sw.) Cooke.

Kniep (*36*) in 1919 proved the existence of two sexes in the sporidia of the anther smut, *Ustilago violacea* Pers. Descendants of one sporidium would not fuse with each other nor with other members of the same sex. Only when the two sexes were brought together did fusions occur. He found different physiologic forms of the smut on different host plants and learned that sporidia of one physiologic form would fuse with those of another. Goldschmidt (*31*) carried further the work of hybridizing physiologic forms of the anther smut and made genetic studies of the progenies.

Dickinson (*22*) showed that in *Ustilago levis* (K. and S.) Mag. and *U. hordei* (Pers.) K. and S. the sporidia were of two sexes. Inoculation with one sex produced no infection. Inoculating with both

produced 91 per cent of infection. Even when sporidia of one sex of *U. hordei* and of the other sex of *U. levis* were used, infection followed. Of the four first-formed sporidia from a single chlamydospore, two were found to be of one sex and two of the other. Cultural differences were found in the four strains from one chlamydospore, and it was determined that segregation of the cultural characters could take place in either of the two reduction divisions.

Stakman and Christensen (*54*) and Stakman, Christensen, and Hanna (*55*) found heterothallism in *Ustilago zeae* (Berkm.) Ung. They found many physiologic forms of the smut and noted the origin of new forms by mutation. Hanna (*33*), continuing the work on *U. zeae*, found that monosporidial cultures could infect the host weakly but produced no galls. When a corn plant was inoculated with two cultures of opposite sex, fusion occurred just after entry, and later galls were formed. The sporidia from one chlamydospore were of two or, less commonly, four sexual groups. Two pairs of factors at least were involved. All geographic races tested showed the same sexual groups. Mutants which appeared in monosporidial cultures showed altered sexual behavior.

Of special interest is the recent announcement by Craigie (*18*, *19*, *20*) of heterothallism in rusts. *Puccinia graminis* Pers. and *P. helianthi* Schw. have been proved experimentally to be heterothallic, and there are indications of heterothallism in *P. coronata* Cda., *P. pringsheimiana* Kleb., and *Gymnosporangium* sp. Monosporidial or simple pustules produced pycnia, but very few produced aeciospores. Of the bisporidial or compound pustules nearly half produced open aecia. When pycniospores of one sex were transferred to a monosporidial infection of opposite sex, aecia developed. In nature this transference is effected by insects which feed on the nectar. More recently Hanna (*34*) has found pycniospores germinating, the largest seen being 15μ long.

The existence of heterothallism and the genetic behavior of the strains of a rust are determined by greenhouse studies. Details of the history of heterothallism as it takes place in the host plant can be learned only by means of the microscope. On this account, a cytological study of the pycnia and aecia of a heterothallic rust, *Puccinia graminis*, was undertaken.

MATERIAL AND METHODS

Teliospores of *Puccinia graminis* Pers. on *Agrostis alba* L. and a supply of barberry plants were obtained from H. B. Humphrey. Teliospores on *Triticum vulgare* Vill. and *Hordeum jubatum* L. were received from W. P. Fraser. The majority of the barberry plants were grown in the greenhouse. A few were planted in the field.

Tall glass tumblers or glass vases were used in inoculating. A line was filed around the bottom of the tumbler or vase and the bottom removed. The rusted straw was soaked in rain water for an hour or two. Watch crystals or halves of small Petri dishes were filled with mud, bits of the rusted straw pressed into the mud with the rusted side exposed, and the surface sprayed with an atomizer filled with rain water. Then the tumbler was lined with one or two layers of wet paper towel, the base of the stem of the plant surrounded with wet sphagnum, the tumbler placed over it, and the mud-filled Petri

dish inverted over the top of the tumbler for a lid. Another layer of wet paper towel was folded over the top and held down with a rubber band. If the plant was small, the whole plant was covered; if larger, only the growing tip of one branch was inclosed. The whole was placed under a greenhouse bench and kept moist for 48 hours and then uncovered and replaced on top of the bench. Cages made by covering wire frames with fine-mesh "tarlatone"[4] were placed over the plants to exclude insects.

The first flecking of the leaves appeared on the fourth to the sixth day, varying with the weather. The infections were studied and counted several times a week and greenhouse records kept.

Material was fixed daily from the time of inoculation until the infections were about 3 weeks old and at longer intervals from then until the infections and the leaves bearing them died. The fixing solutions used were Flemming's medium and weak solutions, chrom-acetic-urea mixtures, and chrom-acetic-formalin mixtures. Of these, Flemming's medium solution proved most trustworthy. The material was washed, dehydrated, and embedded in 50° paraffin. Sections were cut 10μ thick. Three methods of staining were used—Flemming's triple stain, gentian and iodine, and safranine and methylene blue. The last proved most useful.

INVESTIGATIONS

ENTRANCE AND DEVELOPMENT OF MYCELIUM

The youngest material available for study was fixed after two days in the inoculating chamber. During that time some of the teliospores germinated and produced and released sporidia which fell upon the host, usually on the leaves, where they germinated, and the resultant rudimentary fungi entered the epidermal cells. Some of the sporidia failed to germinate, probably due to local deficiency in moisture, but they had shrunk and deteriorated to such an extent that cell contents were indistinguishable.

For uniformity and clearness all drawings are oriented in the plates as the tissues drawn are oriented in the leaf, i. e., with upper epidermis at the top of the drawing or with lower epidermis at the bottom.

The germinating sporidia enter the host by passing directly through the outer wall of epidermal cells. There are no stomata on the upper surface of barberry leaves. In Plate 1, A, the earliest stage available, entry is complete. The fungus entered through the outer epidermal wall at *a*, widened into a sacklike mass in the interior of the cell, then pushed out a hypha, *b*, toward the inner surface of the host cell. There are two nuclei in the central part. If one may judge by what occurs in *Puccinia coronata*, where earlier stages have been studied (unpublished data), the division of the original sporidial nucleus occurs before entry. A lobe of the host nucleus, *c*, lies in contact with the hypha.

This primary hypha elongates, usually curving toward the central region of the host cell. In Plate 1, B, it is a curved, sausage-shaped cell, *b–d*, still binucleate, and connected by a slender beak at *b* to

[4] An open-woven cotton cloth somewhat in the nature of cheesecloth, and nearly as coarse as mosquito netting.

the empty, collapsed, sporidial wall, *a*. The host nucleus is in attendance at *c*.

Nuclear and cell divisions soon follow. In Plate 1, C, the discarded sporidial wall, *a*, lies near the point of entry, *b*. The hypha, *b–c*, is divided by two septa into three cells. The proximal cell, i. e., that nearest the point of entry, is binucleate, the others uninucleate. In Plate 1, D, on the contrary, it is the central cell, *b*, that contains two nuclei. This is less common. Here (pl. 1, D) the hypha has broken loose from the outer epidermal wall and lies free in the interior of the host cell.

The majority of the young rust fungi are in the 3-cell stage on the second day. Comparatively few have developed beyond it. The third day, however, brings marked progress.

The next step may be just growth in length of the primary hypha, or growth in length accompanied by the formation of branches. In Plate 1, E, drawn in outline only, the unbranched hypha is 27μ long and is composed of five cells. This is an extreme case. The type shown in Plate 1, F, is far more common. Here, while the primary hypha is still short and consists of but three cells, its first cell, *a*, has pushed out a short thumblike branch, *b*, directed toward the inner epidermal wall.

In the cell drawn in Plate 2, A, the situation is more complicated. There have been two independent entries into the same epidermal cell. In *c*, presumably the older, the primary hypha grew, became septate, formed several branches, and then deteriorated. In *a*, on the contrary, grown from a sporidium at *b*, a short, thick primary hypha has formed, consisting of four bulging, uninucleate, unbranched cells. Growth in length may have been hindered in this case by the presence of the older infection.

Ordinarily each cell of the primary hypha in turn gives rise to a branch, beginning with the oldest cell. In only one instance, so far seen, did the distal cell branch first. The branches arise on the convex side of the curved hypha at the distal end of each cell. Plate 2, B, shows this. Entry occurred at *j*, the hypha grew in a curved line to *h*, then doubled back to *e*. The oldest cell, *a*, gave rise to a branch, *b*, which passed through into the next cell, forming the intracellular hypha, *c*. The parent cell, *a*, is dead. The second cell, *d*, branched in the same fashion, forming the intracellular hypha, *f*. The original cell, *d*, is dying. In the third cell, *i*, which is still vigorous, the nucleus has divided and one daughter nucleus has passed out into a short branch at *g*. In the fourth cell the only indication of branching is a rounded knob at its distal end on the convex side, at *h*. The fifth cell is unbranched.

EXPLANATORY LEGEND FOR PLATE 1

(The teliospores of *Puccinia graminis* used in growing these infections were obtained from three different hosts. The source of each drawing is indicated by abbreviations, as follows: *Agrostis alba* = (Agr.); *Triticum vulgare* = (Trit.); *Hordeum jubatum* = (Hor.).)

A.—(Hor.) Two-day infection. The fungus, *a–b*, has just entered the epidermal cell. The host nucleus is at *c*. × 1,130.

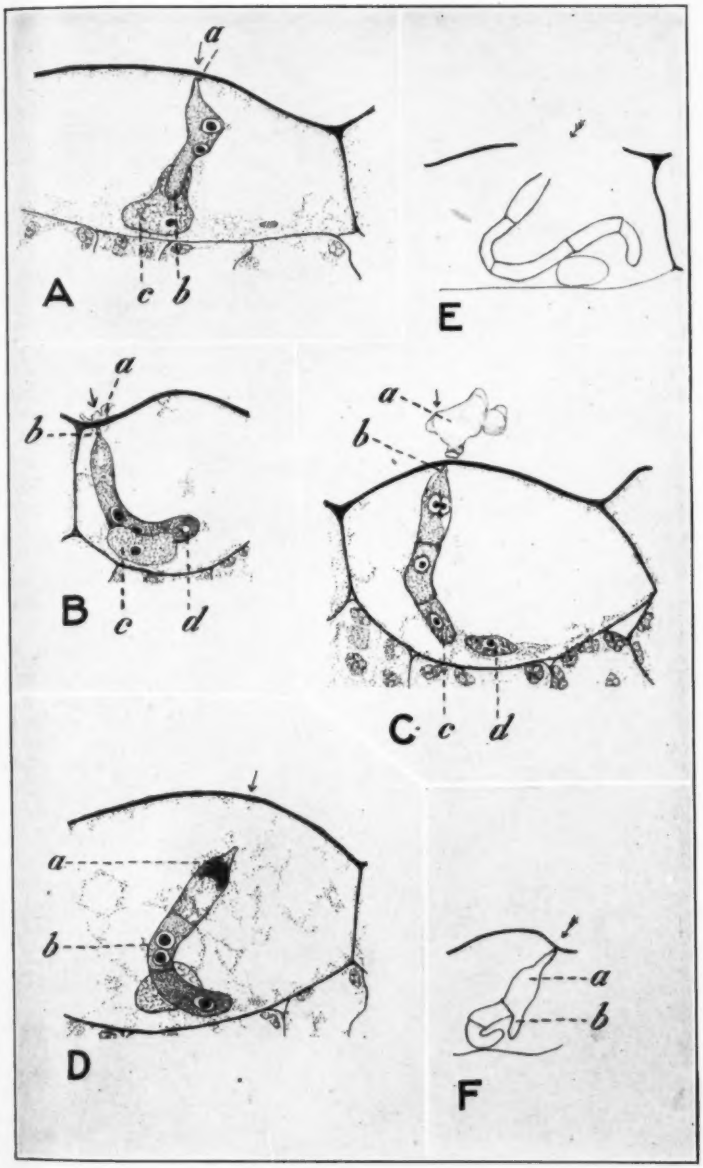
B.—(Hor.) Two-day infection. Still a single binucleate cell, *b–d*. Empty sporidial wall at *a*. Host nucleus at *c*. × 1,130.

C.—(Hor.) Two-day material. Empty sporidial wall at *a*. Primary hypha, *b–c*, now 3-cell. Proximal cell binucleate. × 1,130.

D.—(Hor.) Two-day infection. Three-cell primary hypha, *a–b*, free in epidermal cell. Central cell, *b*, binucleate. × 1,130.

E.—(Hor.) Three-day infection. Primary hypha 5-cell, unbranched. × 1,130.

F.—(Hor.) Three-day infection. Three-cell primary hypha. Proximal cell, *a*, branched at *b*. × 1,130.



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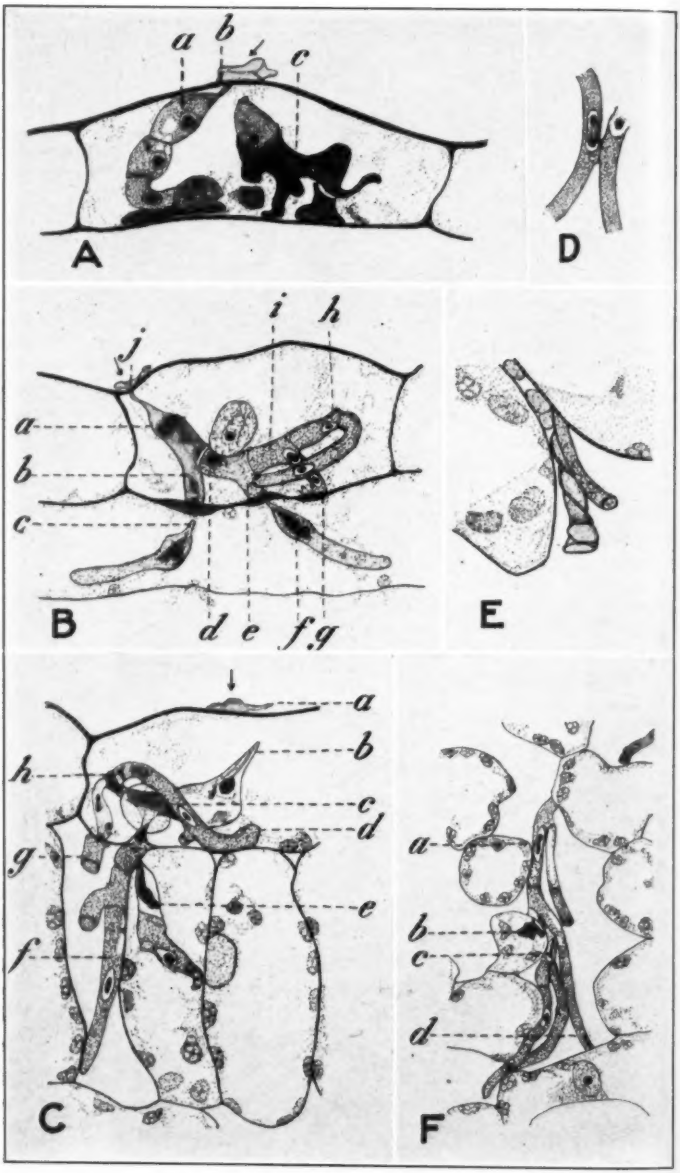

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It is of interest that a swelling occurs in the inner epidermal wall (pl. 2, B) at each of the three points of contact with the fungus. This has not been observed elsewhere. This infection lies over a vein, and it may be that cell walls here differ chemically from palisade walls. Whether over veins or palisade cells, the actual opening in the wall through which the hypha passes is too minute to be seen.

In the infection shown in Plate 2, C, the beaklike entry tube of the hypha, *b*, lies loose in the interior of the cell, and the hypha has grown to the side wall at *h*, then doubled back to *d*, forming a loop. The host nucleus, *c*, has been left transparent in the drawing to show the fungus beneath it. All of the cells of the primary hypha have branched, and the branches have made their exit into the subepidermal area. Two of these, *f* and *g*, occur in this section, the rest in adjoining sections. Five and even six secondary hyphae may form from one primary hypha.

The rate of growth and the form of these secondary hyphae differ markedly according as they emerge into an intercellular space or into a palisade cell. The entry tube of an intracellular hypha is beaklike in shape (pl. 2, B, *c*, *f*; C, *e*; pl. 3, A, *h*), like the entry tube of the primary hypha (pl. 2, B, *a*; C, *b*; pl. 3, B, *a*). Hyphae passing into intercellular spaces, on the contrary, broaden at once to full size (pl. 2, C, *g*; pl. 3, B, *d*; pl. 3, A, *b*). The first intercellular hyphae (pl. 2, C, *f*, *g*) are vigorous and shapely and quite like the later mycelium. The growth within palisade cells, on the contrary, presents all gradations from simple haustoria to 3-cell or 4-cell hyphae not unlike the primary hypha in character (pl. 2, C, *e*). The simplest are 1-cell, unbranched, and uninucleate; others have the tips forked and curved (pl. 3, A, *c*); and some are septate and have two or three cells. The latter, like the primary hypha, are often irregular in form and are usually short-lived. A few are fully mycelial in character and grow directly through palisade cells and out into the air spaces of the mesophyll.

Once free of the compact palisade layer, the mycelium is regularly intercellular in character. Later haustoria (pl. 3, A, *h*, *i*) may be either branched or unbranched and are commonly, but not always, 1-cell.

Plate 3, B, still from 3-day material, shows another case of two primary hyphae within the same host cell. It is not rare to find four or five within a range of two or three epidermal cells. On the basis of heterothallism, a rust plant would be either (+) or (−). It seems highly unlikely that all the members of a group of four or five would be (+) or all (−). The question at once arises as to whether a (+) and a (−) hypha can fuse, giving rise directly to sporophytic mycelium. The search for this has yielded nothing conclusive. In one case (pl. 2, D) in the margin of an older mycelium, two hyphae

EXPLANATORY LEGEND FOR PLATE 2

A.—(Hor.) Three-day infection. Two primary hyphae, *a* and *c*, in one epidermal cell. Older one, *c*, dying. × 1,130.

B.—(Hor.) Three-day infection. Empty sporidial wall at *j*. The primary hypha grew from *a* to *h* and back to *e*. Proximal cell, *a*, branched at *b*, forming subepidermal hypha, *c*. Second cell, *d*, formed hypha at *f*. Third cell, *i*, formed branch at *g*. × 1,130.

C.—(Agr.) Three-day infection. Sporidial wall at *a*. The primary hypha grew from *b* to *h*, then back to *d*. Host nucleus at *c*. Branches formed intercellular hyphae at *f* and *g* and an intracellular hypha at *e*. × 1,130.

D.—(Agr.) Detail from a 10-day infection. Two hyphae anastomosed. × 1,400.

E.—(Agr.) From a 10-day infection. Two hyphae twisted around each other. × 1,400.

F.—(Agr.) Detail from 4-day infection. Cells uninucleate. Elongated nuclei at *a*, *c*, *d*. Haustorium at *b*. × 730.

met and seemingly anastomosed, but there was no proof of transfer of nuclei. Several times two hyphae have been found twisted tightly into a rope (pl. 2, E), but here, too, there is no proof that binucleate mycelium arises from it. So far as noted, the cells of young interlacing mycelia are strictly uninucleate, and even in older mycelia there is at present no unmistakable proof that the sporophytic mycelium has arisen directly by fusion of gametophytic hyphae.

Plate 3, B, illustrates another point. One branch, *c*, from a primary hypha, has emerged upon the outer surface of the leaf and formed a considerable growth there. Two other similar instances have been seen. The extreme humidity of the inoculation chamber probably facilitates this. It is doubtful whether it occurs often in nature.

Once the mycelium has reached the spongy mesophyll of the leaf, it spreads freely. The rate of development varies with conditions. Early in May, 4-day infections in the greenhouse consist of vegetative mycelium. In June, 4-day infections have begun reproductive activities and bear all stages up to half-grown pycnia. The difference may be due to the increased light and higher temperature at the later date.

Plate 3, A, represents the central section through a 4-day infection fixed on May 1. The remnants of the rust in the epidermis are too disintegrated to permit an interpretation. It is not certain whether a single primary hypha, *d*, passed through the side wall of the cell at *f* and formed a growth in the adjoining epidermal cell, or whether there were two independent primary hyphae. The former seems more likely. In the first palisade cell entered, the rust formed a several-cell hypha, *c*, which is dead, and its host cell is also shrunken and dying. The adjoining palisade cell was entered near *a* (the connection was lost in sectioning), the hypha grew to the inner end of the cell, branching on the way, and passed out at *b*, into intercellular spaces. From here, and from *e*, where the fungus passed directly from the epidermis into intercellular spaces, the mycelium has spread through the air spaces, covering an area 140μ in diameter. The older intercellular hyphae, at *b* and *g*, are drained of stainable content, the materials having presumably passed on toward the growing tips, *j*, *k*.

The cells of the hyphae are uninucleate so far as noted (pl. 3, A, *j*, *k*; pl. 2, F). When hyphal cells are growing rapidly, both the nucleus as a whole and the nucleolus within it may be elongated. (Pl. 2, F, *a*, *c*, *d*.)

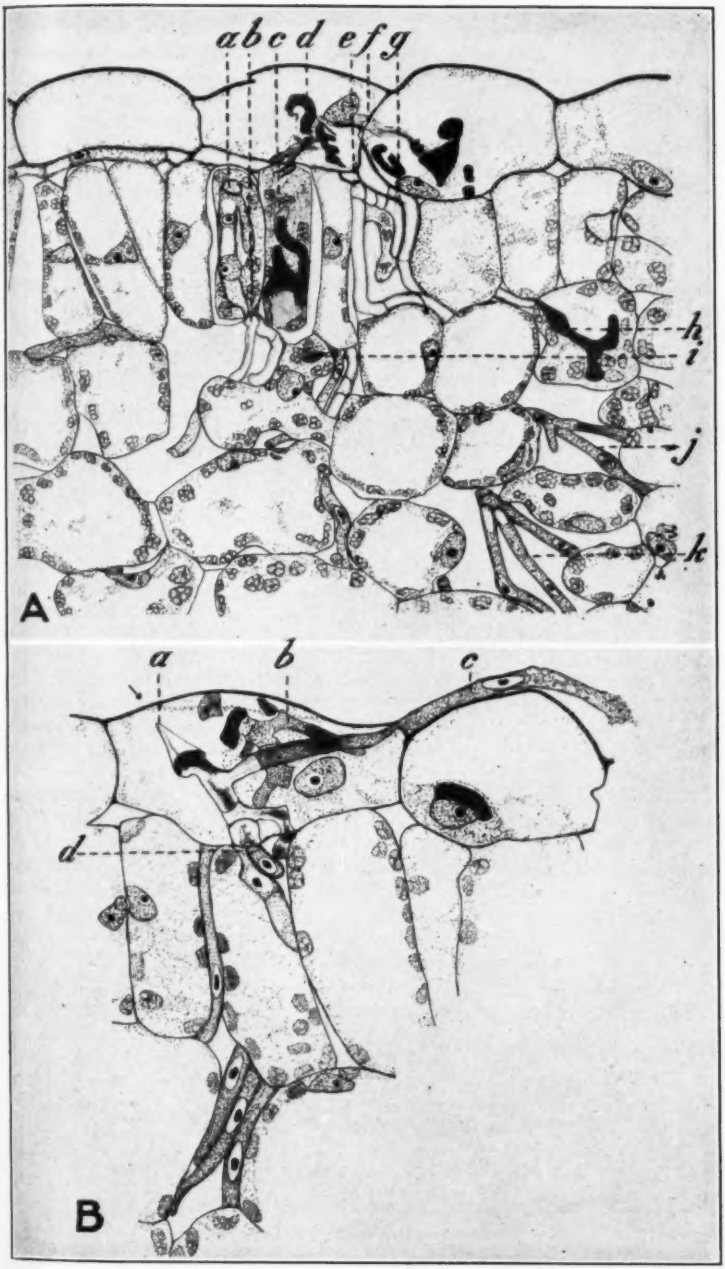
DEVELOPMENT OF THE PYCNIUM

An early stage of pycnial development is represented in Plate 4, A. Hyphae with dense contents (pl. 4, A, *b*, *c*) force a passageway up between palisade cells and between the epidermal and palisade layers. These hyphae branch freely, forming a subepidermal mat in which at first no organization is evident. Soon, however, they begin to converge toward a central point, or if the area is unusually large there may be two centers of development. In Plate 4, A, this convergence is just beginning. Hyphae are turning toward *a*. In Plate 4, B, a slightly later stage drawn to the same scale, the centering

EXPLANATORY LEGEND FOR PLATE 3

A.—(Agr.) Median section of 4-day infection. Disintegrating primary hypha, *d*, *f*. Dying intracellular hypha, *c*. Living intracellular hypha at *a* passes into intercellular space at *b*. Intracellular hypha at *h*. Intercellular mycelium at *e*, *g*, *j*, *k*. Haustorium at *i*. × 730.

B.—(Agr.) Three-day infection. Two primary hyphae, *a*, *b*, in one epidermal cell. Intercellular hyphae at *d*. Hypha on outer surface of host at *c*. × 1,130.



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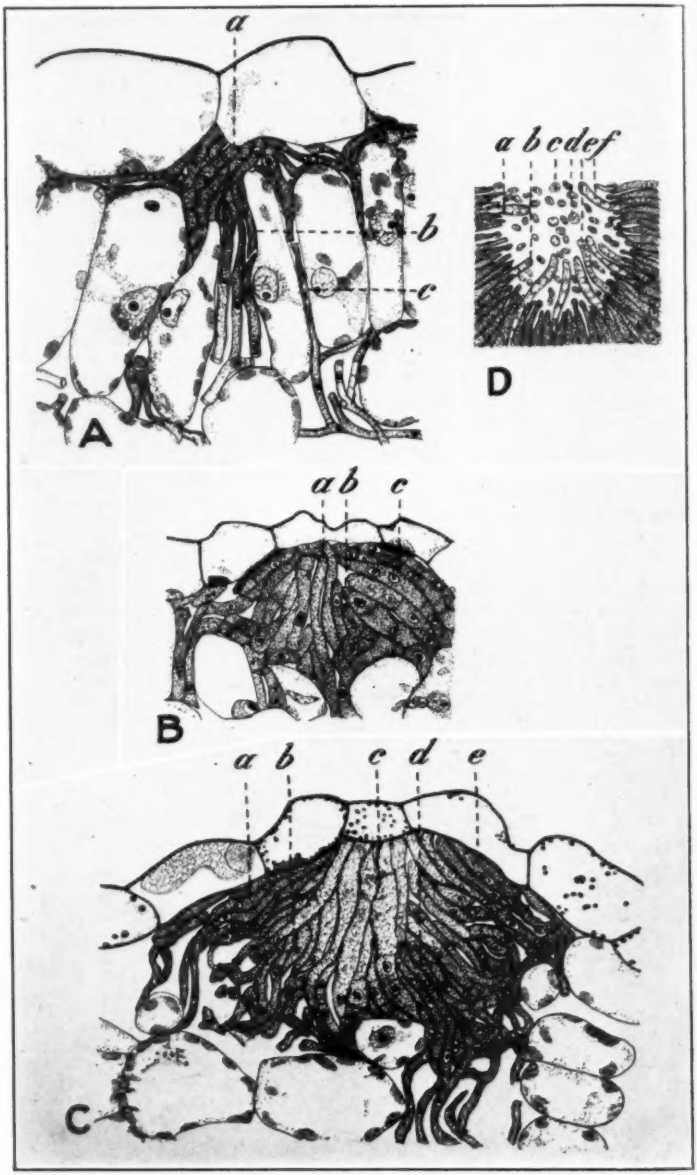

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is more evident. By proliferation the few original hyphae have multiplied, and from all sides they are aimed at the common point, *a*. The cells are regularly uninucleate. A few (pl. 4, B, *b*, *c*) contain two nuclei, but these are the rapidly growing terminal cells of hyphae, and nuclear division has not yet been followed by cell division.

A later stage (from 5-day material) is represented in Plate 4, C. This subepidermal mass now has the form of a thick biconvex lens. The raised epidermis, out of its normal food relations with the mesophyll tissue and stretched by the growing fungous mass beneath it, is somewhat flattened and is dying. The hyphae have multiplied by further branching and are well centered. The uppermost hyphae, *a*, *e*, running just beneath the epidermis and parallel to it, have remained slender and are dense in content. These will later give rise to paraphyses. The hyphae at the center beneath *c* are upright and columnar, and their great size and turgor and their vacuolated cytoplasm suggest that they are exerting force in lifting the epidermis and are serving as buffers against its pressure. Groups of smaller hyphae (pl. 4, *C*, *b*, *d*) are forcing their way inward from the margin. These are the first of the pycniosporophores which will later produce the spores.

The number of pycniosporophores now increases rapidly. Each arises from a marginal hypha and grows inward. These soon form a continuous compact marginal layer, and the whole pycnium arches out as new pycniosporophores are interpolated between the older. It soon forms a rounded mass, displacing mesophyll cells as it grows. This expansion of the pycnium releases the buffer cells from their pressure against the epidermis. In Plate 6, B, is drawn at high magnification the central group of buffer cells, *b*, and the inner wall of an epidermal cell, *a*. The buffer cells are no longer pressed tightly against one another and against the epidermis. They are somewhat lax and their tips lie free in the center of the pycnium. As before, their cytoplasm is vacuolate and the nucleus of each is far back from the tip. Pycniosporophores are encroaching upon their space at *c* and *d*.

In Plate 5, A, is a somewhat later stage. The tips of the growing paraphyses have met at the apex and turned outward, exerting pressure against the epidermis at *a*, where they will soon break through. It is to be noted that there is a single nucleus at the growing tip of each paraphysis. Surrounding the main body of the pycnium are one or two layers of short cells forming a more or less definite pycnial wall. Numerous pycniosporophores extend inward and upward from this wall, curving toward the future ostiole. The buffer cells, *b*, in this preparation are present but are stained too faintly to be drawn in detail.

By the sixth day, under favorable conditions, the first pycnia have matured and opened and are shedding spores through the ostiole or apical opening. Plate 5, B, represents a newly opened pycnium. It has expanded farther, making space for itself by crushing adjoining

EXPLANATORY LEGEND FOR PLATE 4

A.—(Trit.) Four-day infection. Hyphae forcing passageway up between palisade cells at *b*, *c*, to start a pycnium at *a*. × 730.

B.—(Hor.) Slightly older pycnium in 6-day infection. Hyphae converging at *a*. Young pycniosporophore at *b*. Young paraphysis at *c*. × 730.

C.—(Trit.) Larger pycnium from 5-day infection. Buffer cells at *c*, young pycniosporophores at *b*, *d*, and paraphyses at *a* and *e*. × 730.

D.—(Hor.) Center of mature pycnium from 9-day infection. Buffer cells at *a*, *b*, *c*, *d*, *e*, *f*. × 730.

chlorophyll cells, *e*, *f*, and by lifting and flattening the epidermis at the sides, *a*, *c*, and rupturing it at the top, *b*. From its outer wall or cortex the pycniosporophores extend inward, and at their slender tips pycniospores are formed and set free into the central cavity.

Details of the development of pycniosporophores and spores are presented in Plate 6, E. The young pycniosporophores found in the pycnium before it opens (pl. 6, E, 1) is thick and often curved, and tapers to a rather blunt tip. It contains dense cytoplasm and a single nucleus. As it grows (pl. 6, E, 2) the nucleus divides and sometimes, but not always, a septum near the base gives rise to a short stalk cell (2, *a*, and 4, *d*). The terminal cell continues to grow in length. Pycniosphorophores are crowded together, and details of shape, length, and thickness vary with the available space. In 3 the primary nucleus has divided. One of the two daughter nuclei (3, *b*) has remained near the base and is growing rapidly; the other (3, *a*) has not grown and is migrating toward the swelling tip of the cell. A little later this nucleus reaches the tip (4, *a*), and the basal nucleus divides again (4, *b*, *c*). In Plate 6, E, 5, the spore, *a*, has been abscissed, the tip of the cell, *b*, is reshaping to form a second spore, and another nuclear division has provided the extra nucleus, 5, *c*, which has begun to move toward the tip. This process continues until, as in Plate 6, E, 6, there is a row of free spores, *a*, *b*, *c*, beyond the tip, a fourth forming at *d*, and provision for a fifth in the nucleus, *e*. This procedure keeps on indefinitely. The first spores emerge through the ostiole soon after the opening of the pycnium (pl. 5, B, *b*), doubtless carried out in the swelling slime in which they are embedded.

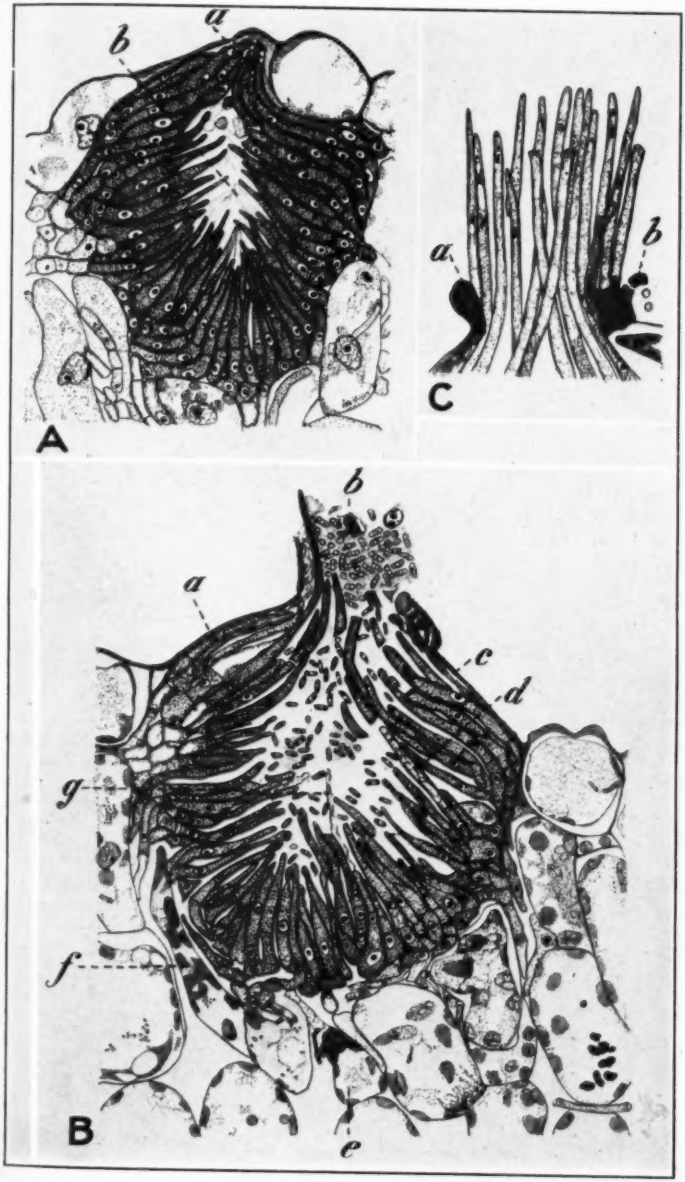
The buffer cells, which filled the central area in the younger pycnia (pl. 4, C, *c*, and pl. 5, A, *b*), are now inconspicuous, vacuolated cells, extending somewhat farther into the central cavity than the pycniosporophores. Plate 4, D, represents the central cavity of a newly opened pycnium. The buffer cells (seen in side view in pl. 4, D, *a*, *b*, *e*, and in cross-section at *c* and *d*) no longer form a compact group; they have been separated by the pycniosporophores that squeezed in between them. They are still, however, long, coarse, blunt-tipped cells with vacuolated cytoplasm. In Plate 6, C and D, are drawn separately at high magnification buffer cells in different stages of deterioration. In C is drawn a buffer cell, *a*, with a pycniosporophore, *b*, alongside. The buffer cell is longer, coarser, and has a blunter tip and thinner content. D, *b*, represents a dying, and D, *c*, a dead buffer cell. The dotted line, *a*, in each is at the level of the tips of the pycniosporophores. At *g*, in Plate 5, B, is a withered buffer cell, and at *d* is one that is still turgid. The time at which the buffer cells disappear varies greatly. A few are still present in pycnia of 20-day infections. It would appear, then, that the buffer cells serve only the transient purpose of helping to lift the epidermis above the young growing pycnium. So far as noted they produce no spores, although they may be present long after spore production has begun.

EXPLANATORY LEGEND FOR PLATE 5

A.—(Agr.) Nearly mature pycnium from 7-day infection. Paraphyses turned outward at *a* to break epidermis. Numerous pycniosporophores. Buffer cells at *b*. × 730.

B.—(Trit.) Newly opened pycnium in 6-day infection. Paraphyses at *a*. Crushed epidermis at *c*. Buffer cells at *d* and *g*. Spores emerging at *b*. Crushed mesophyll cell at *e* and *f*. × 730.

C.—(Trit.) Six-day infection. Tuft of young paraphyses between broken epidermal cells at *a*, *b*. × 730.



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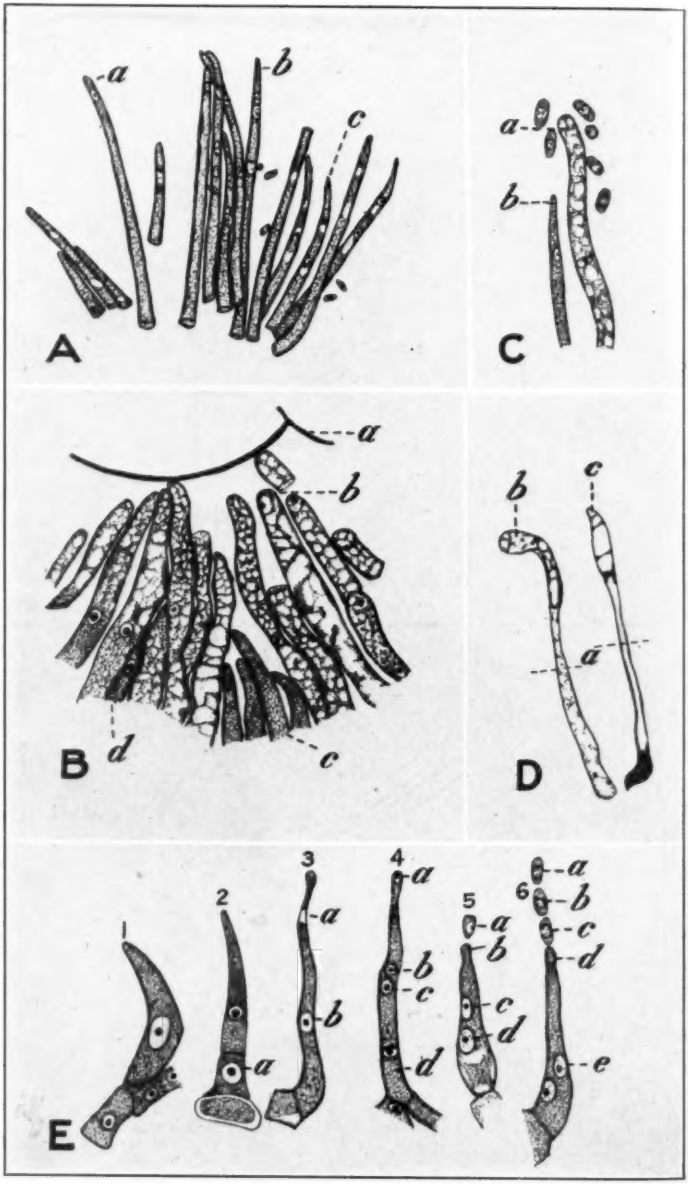

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The paraphyses, which in an earlier stage (pl. 5, A, *a*) were about to rupture the epidermis, have now (pl. 5, B, *b*) broken through, forming the ostiole. They soon elongate into a brush of stiff-looking hyphae, each tapering to a point (pl. 5, C; 6, A) containing living cytoplasm and 1, 2, or even 3 nuclei.

Pycnia grow considerably after the first maturity, and older pycnia that have produced spores for a week or more sometimes open wider. In extreme cases the ostiole nearly equals in size the cross diameter of the pycnium.

The number of spores produced is enormous. The pycnia of an infection maintain a good-sized drop of "pycnial exudate," a golden brown, sirupy liquid filled with spores, on the upper surface of the leaf throughout their active period. When two or more infections are close together the drops often coalesce into one still larger drop.

HETEROTHALLISM

The course of development of all infections of *Puccinia graminis* studied is essentially similar up to the time when the pycnia mature. From this point on, their progress diverges into one or other of two channels leading to very different ends. Some of the infections produce aeciospores and others remain sterile.

If *Puccinia graminis* is heterothallic and there are only two sexual groups, each plant of the gametophyte generation is either (+) or (−). Neither a (+) nor a (−) strain when isolated can form diploid mycelium or aeciospores. Only when (+) and (−) are brought together can fertile aecia form.

A count was made of all the infections on a plant 32 days after inoculation. This is long after the first appearance of open aecia. The plant was left untouched during the growth of the infections. The infections were classified as (1) singles (i. e., isolated infections) without open aecia, (2) singles with aecia, (3) doubles (i. e., two infections in contact) without open aecia, and (4) doubles with aecia. The count showed 37 singles without open aecia, 2 singles with open aecia, 14 doubles without and 15 doubles with open aecia. Theoretically, the singles and 50 per cent of the doubles should remain sterile, and the other 50 per cent of the doubles should produce aeciospores. The above count fits the expectation closely—only the two fertile singles are out of line. Counts of other infected plants have been made. Some of them did not come so close to the theoretical expectation, especially when infections were somewhat crowded. In these cases there were more infections with aecia than would be expected.

A few days later (36 days after inoculation) the infections on the plant described above were fixed. Sections through typical sterile

EXPLANATORY LEGEND FOR PLATE 6

A.—(Trit.) Tuft of young paraphyses from pycnium of 6-day infection. A paraphysis may contain 1 (*a*), 2 (*c*), or 3 (*b*) nuclei. × 730.

B.—(Trit.) Eight-day infection. Group of buffer cells from center of half-grown pycnium. Epidermal wall at *a*. Buffer cells at *b*. Young pycniosporophores at *c*, *d*. × 1,460.

C.—(Trit.) Eight-day infection. Buffer cell, *a*, and pycniosporophores, *b*, from open pycnium. × 1,460.

D.—(Trit.) Eight-day infection. Dying buffer cell, *b*, and dead buffer cell, *c*, from mature pycnium. Level of tips of pycniosporophores at *a*. × 1,460.

E.—(Trit.) Details showing development of pycniosporophores and spores. 1. Young pycniosporophores. 2. Older, with stalk cell at *a*. 3. Nucleus divided. One daughter nucleus, *b*, at base; the other, *a*, moving toward tip. 4. Nucleus, *a*, at tip where spore is forming; basal nucleus, divided, *b*, *c*. 5. Spore freed at *a*; tip of pycniosporophore reshaping to form second spore; two nuclei below, *c*, *d*. 6. Three spores, *a*, *b*, *c*, freed, a fourth forming at *d*, and an extra nucleus at *e*. × 1,460.

and fertile infections from this plant are represented semidiagrammatically in Plate 7, A and B. Plate 7, A, is a median section through an isolated infection. The infected area of the leaf is greatly hypertrophied, as can be seen by comparing the normal thickness of the leaf at *e* with its thickness in the infection. In spite of the great age of the rust, pycnia are numerous and still active. There was an abundant pycnial exudate on the living leaf, some of which adhered through the processes of fixing, washing, etc., and is still present. (Pl. 7, A, *a*, *c*, *d*.)

In addition to the pycnia, other fungous structures have formed near the lower surface of the leaf. (Pl. 7, A, *f*, *g*, *h*, *i*, *j*, *k*, *l*.) These are nearly spherical and consist of an outer shell of small-celled pseudoparenchyma and a central mass of larger rounded cells. They are dead or nearly dead. A cytological examination of many such isolated infections shows that these structures are of regular occurrence. There are from 3 or 4 up to 2 dozen or more in each infection. They are sterile aecia.

Plate 7, B, is a section through two adjoining infections separated only by a vein at *c*. Here, too, the leaf tissue is hypertrophied. Pycnia are present (pl. 7, B, *a*, *b*, *d*, *e*, *f*), but they have ceased to function, and the remaining bits of exudate are dried and dead. Here, too, a row of fungous structures has formed in the leaf near the lower surface (pl. 7, B, *g*, *h*, *i*, *j*, *k*, *l*), but with one exception (*i*) they have developed into normal aecia and are shedding spores freely. The vein between these two infections is impenetrable to mycelium, but the pycnial exudate of the two may easily have been mixed.

HISTORY OF ISOLATED INFECTIONS

Material was available for a study of the processes leading to the sterile condition shown in Plate 7, A.

The newly opened pycnium and the young paraphyses have been described. Within and around the pycnium, cells are uninucleate. (Pl. 5, B.) A young paraphysis about to break through the epidermis contains a single nucleus near its apex. (Pl. 5, A, *a*.) Soon after its emergence the nucleus may divide. Some of the paraphyses in the tuft in Plate 5, C, contain more than one nucleus. In the brush of paraphyses in Plate 6, A, a single cell may contain 1 (pl. 6, A, *a*), 2 (*c*), or even 3 (*b*) nuclei. So far as noted, the emergent part of the paraphysis is nonseptate. Both of these tufts occur in 6-day infections. The pycnia have been open only a few hours.

The paraphyses on the pycnia of older sterile infections show the same nuclear content. In Plate 7, C, drawn from a pycnium in a 14-day infection, the paraphyses and their nuclei are somewhat larger, but they still contain two or, less commonly, three nuclei.

In Plate 7, D, is drawn the edge of a pycnium, *a*, and mycelium adjacent to it, *b*, *c*, from a 10-day infection. The pycniospores of the infections on this leaf were mixed a few hours before the material

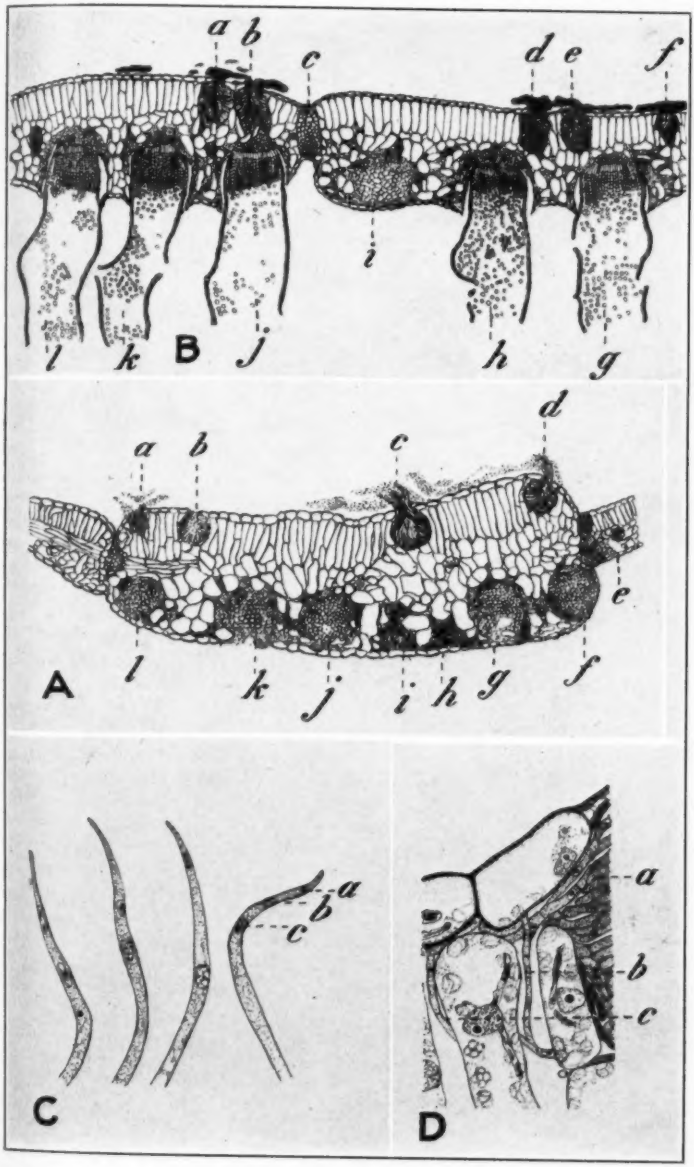
EXPLANATORY LEGEND FOR PLATE 7

A.—(Agr.) Semidiagrammatic drawing of 36-day sterile infection. Living active pycnia at *a*, *b*, *c*, *d*. Sterile dead aecia at *f*, *g*, *h*, *i*, *j*, *k*, *l*. Healthy leaf tissue at *e*. × 40.

B.—(Agr.) Diagram of two fertile infections separated by a vein at *c*. Dead pycnia at *a*, *b*, *d*, *e*, *f*. Fertile aecia at *g*, *h*, *j*, *k*, *l*. Sterile aecium at *i*. × 40.

C.—(Hor.) Paraphyses from pycnium of 14-day sterile infection. Paraphysis contains two or sometimes three nuclei (*a*, *b*, *c*). × 730.

D.—(Agr.) Margin of a pycnium, *a*, and adjacent mycelium, *b*, *c*, from a 10-day sterile infection. × 640.



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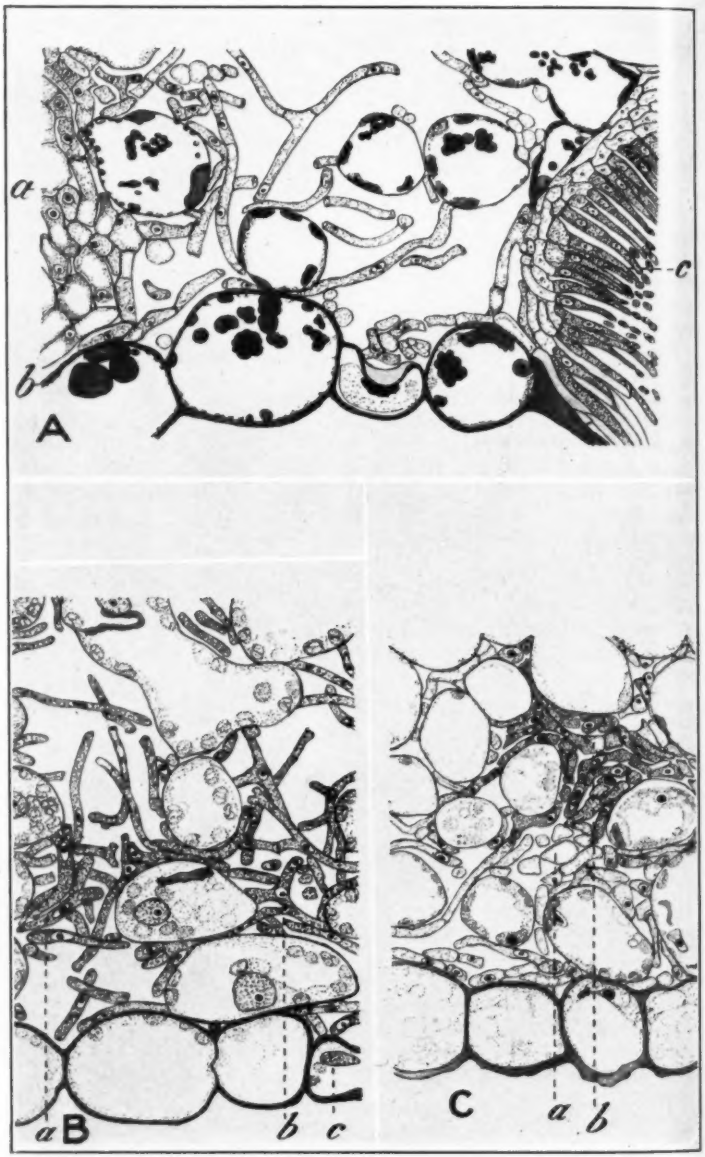

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was fixed. One or two of the other pycnia of this infection give evidence of it, but in this pycnium and the mycelium around it all fungous cells are uninucleate, so far as noted.

In Plate 8, A, from a 9-day sterile infection, is drawn the edge of a pycnium, *c*, the edge of a young aecium, *a*, and the area between. Pycnia commonly form beneath the upper surface of the leaf, but this one, as is not rare, opens downward. In the pycnium, the aecium, and the mycelium between the two, the cells are uniformly uninucleate.

The earliest indication of aecia in sterile infections is the formation of denser wefts of mycelium in the large air spaces of the mesophyll tissue near the lower surface of the leaf. Plate 8, B, from a 10-day infection, depicts such a one. The slender hyphae, *a*, *b*, run in all directions between the host cells, branching freely. They are composed of uninucleate cells.

The hyphae in this loose tangle grow and branch rapidly and become interwoven into a solid little mass of pseudoparenchymatous tissue. (Pl. 8, C.) This is early differentiated into an outer half of larger cells with thin contents (pl. 8, C, *a*) and an inner half of smaller cells with dense contents (pl. 8, C, *b*).

The differentiation into areas becomes accentuated with further growth. Plate 9, A, from a 10-day sterile infection, represents semi-diagrammatically, at low magnification, a larger sterile aecium, with its lower region, *c*, of large rounded vacuolate cells, its upper region, *b*, of small dense angular cells, and an outer covering, *a*, surrounding the whole, consisting of several layers of small cells. The fungous mass has crushed or pushed aside mesophyll cells as it expanded. The central region between *b* and *e* is drawn enlarged (same scale as preceding drawings) as Plate 9, B. With one doubtful exception (pl. 9, B, *b*), the cells are uninucleate throughout. The central area between *a* and *c* is composed of cells very rich in cytoplasm, but there is no indication of activity leading to spore formation.

The time of formation of these sterile aecia varies. Rarely, aecia may begin to form before the opening of the first pycnium, but more commonly about the ninth or tenth day. As the mycelium spreads radially, new aecia arise in succession out toward the margin.

In Plate 9, C, is drawn at lower magnification a small specimen of a later stage. The cells are still living but impoverished, the outer and inner regions alike consisting now of nearly empty cells.

Plate 10, A, shows a sterile aecium from a 36-day infection, the age shown in the diagram in Plate 7, A. The whole fungous mass is dead. In the center are greatly enlarged rounded cells, empty and clear. Sometimes a break in this mass leaves an irregular clear area (pl. 10, A, *c*) which may, as in this case, be threaded by fine hyphae, now also dead. Around the outside (pl. 10, A, *a*) is a denser shell consisting of several layers of smaller closely packed hyphae. There were 29 such sterile aecia in the infection from which this one is drawn.

EXPLANATORY LEGEND FOR PLATE 8

A.—(Trit.) Part of a pycnium, *c*, and a young aecium, *a*, and the mycelium between, from a 9-day sterile infection. Lower epidermis at *b*. × 730.

B.—(Agr.) Very young aecium composed of slender hyphae with uninucleate cells, *a*, *b*, from 10-day sterile infection. Lower epidermis at *c*. × 730.

C.—(Hor.) Slightly older aecium from a 9-day sterile infection in a petiole. Differentiated into an inner half (*b*) of small dense cells and an outer (*a*) of larger vacuolate cells. All uninucleate. × 730.

Pycnia in sterile infections continue spore production for a surprisingly long period. In Plate 10, C, is drawn (at low magnification and in outline only) an active pycnium from an infection 51 days old. There are fresh spores in the central cavity and outside. It has undergone considerable modification, however. The pycnium as a whole has enlarged, its outer wall has thickened (pl. 10, C, *b*, *c*), its primary paraphyses (pl. 10, C, *a*) have withered and died, and secondary paraphyses have grown out from a lower level in the pycnial wall. In rare cases, paraphyses may arise from the floor of the pycnium as well as from its side walls. This is accompanied by a widening of the ostiole, until it sometimes equals in diameter the cross diameter of the whole pycnium. The majority of the pycnia in infections over 3 weeks old show these secondary changes in varying degrees.

In Plate 9, D, from a 51-day infection, is drawn part of a group of secondary paraphyses. They are long, slender cells, still living, although the nuclear content is becoming vague. They are predominantly binucleate, so far as can be determined. At one side (pl. 9, D, *a*) are dead remnants of the earlier paraphyses. Plate 9, D, is drawn at the same magnification as the full-grown primary paraphyses in Plate 7, C. A comparison of the two shows that the secondary paraphyses are decidedly longer—almost twice as long—as the primary.

In one lot of material, derived from teliospores on *Hordeum jubatum*, the pycnia of 20-day infections frequently show a different type of secondary change. Here the pycnium stops making spores, and the pycniosporophores elongate, growing to the ostiole and even extending through it, or else becoming snarled up in the central cavity. Meanwhile, at the base of the pycnium there is an irregular type of renewed growth, and there form one or more dense masses of hyphae extending into the deeper tissues of the leaf. Plate 10, B, shows an extreme case of this degenerative secondary growth. At *a* are the withered primary paraphyses. Plate 10, B, *b*, marks the level of the original base of the pycnium, and *c* the deeper secondary growth. The pycnium is hopelessly disorganized and the mass would hardly be recognized as pycnial in origin if intermediate gradations between it and the normal pycnium were not available.

DEVELOPMENT OF FERTILE INFECTIONS

The course of development leading to normal fertile aecia also has been traced.

In Plate 11, C (corresponding to Plate 7, D, of the unfertilized series), is drawn from a 10-day infection the edge of a pycnium, *h*, and the tissues adjoining it. Pycniospores in this case were not mixed by hand, but another infection was so close to this that the pycnial exudate of the two probably was continuous. Above the leaf epidermis (pl. 11, C, *b*) are pycniospores, *a*. Just beneath the epider-

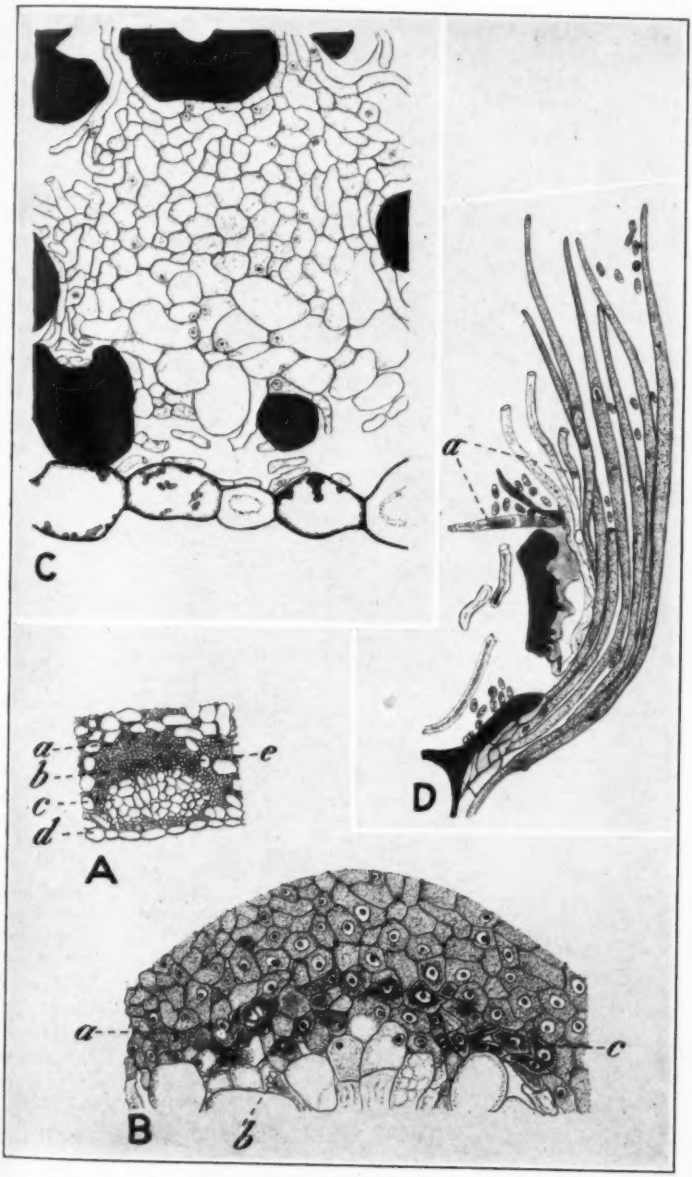
EXPLANATORY LEGEND FOR PLATE 9

A.—(Trit.) Semidiagrammatic drawing of larger aecium from a 10-day sterile infection. Feeding tissue at *a*. Small dense cells from *b* to *c*. Large nearly empty cells at *c*. Lower epidermis at *d*. × 115.

B.—(Trit.) Region between *b* and *c* of A enlarged. Cells denser from *a* to *c*, but all uninucleate. Doubtful binucleate cell at *b*. × 730.

C.—(Trit.) Small sterile aecium from a 14-day infection. Cells impoverished. × 640.

D.—(Hor.) Tuft of secondary paraphyses from a living active pycnium of a 51-day sterile infection. Primary paraphyses now dead at *a*. × 730.



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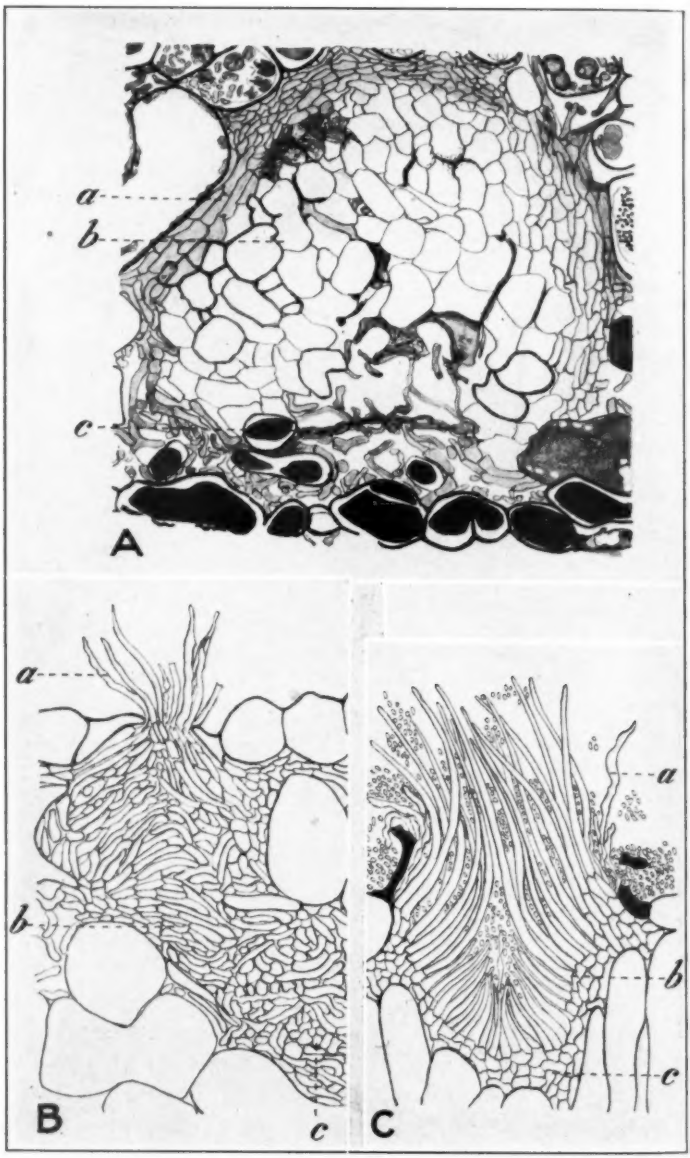

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mis at *g* are the bases of paraphyses, and adjoining these in the wall of the pycnium are fungous cells, *c*, *d*, containing two or three nuclei. Growing downward between palisade cells from this region are slender hyphae (pl. 11, C, *e*, *f*, and in the next section, pl. 11, D, *a*, *b*, *c*), the cells of which contain two or three nuclei.

The fact that diploid hyphae may take their origin at the base of the paraphyses suggests at once that the diplophase may arise through the agency of paraphyses. It is at least hypothetically possible that a pycniospore from another pycnium might be brought in contact with a paraphysis, enter, its nucleus pass down to the base, and there in association with one from the same pycnium start a binucleate hypha; or that a (+) and a (−) pycniospore should become closely associated, the pair of nuclei enter the paraphysis, and move down to the base.

Attempts to determine whether this happens meet with difficulties. As already noted, the native nuclear content of the paraphysis is not uniform. There may be 1, 2, or even 3 nuclei in the extruded part of the young paraphysis before the introduction of spores from other pycnia. (Pl. 5, C; pl. 6, A.) In older isolated infections also the number may be 1, 2, or 3. (Pl. 7, C; pl. 9, D.)

Moreover, pycniospores transferred from another pycnium are indistinguishable from those grown there, and when a dozen spores are seen adhering to a paraphysis it can not be told whether they are native or introduced. The spores are so minute that the actual entry of a spore nucleus, if it takes place, would be difficult to detect.

One lot of material (14-day infection) was fixed a few minutes after transferring pycniospores to it. In several instances paraphyses of the type shown in Plate 12, D, were found in this lot. An occasional paraphysis might contain one or two nuclei (pl. 12, D, *c*), but alongside it would be others containing one or two larger nuclei, *a*, and a group of similarly staining small granules, *b*, which may or may not be nuclei.

In Plate 11, B, is drawn another group of paraphyses from the same lot of material as in Plate 12, D. Here, too, the evidence is inconclusive, but suggestive. In addition to the regular nuclei there are small granules, each surrounded by a narrow clear space, which may or may not be nuclei. A total of four (pl. 11, B, *a*) and even five (B, *b*) is not rare.

Whether or not the paraphyses serve in this capacity, they are probably not the only means by which binucleate hyphae are initiated. In Plate 12, A (from the same infection in which the materials for Plate 11, C and D, were found), is drawn the area from a pycnium, *a*, *e*, on the one side, to the margin of a very young aecium, *h*, on the other. In the pycnial wall the cells are partly drained and the nuclei indistinct. Those that can be determined are uninucleate. At the inner end of the pycnium, however, are several binucleate cells. (Pl. 12, A, *a*, *b*, *c*.) Other similar cases have been seen.

EXPLANATORY LEGEND FOR PLATE 10

A.—(Agr.) Dead haploid aecium from a sterile 36-day infection. Outer cortex of small cells at *a*; larger central cells at *b*; finer dead hyphae at *c*. × 333.

B.—(Hor.) Teratological growth of a pycnium in a 20-day sterile infection. Dead paraphyses at *a*. Original base of pycnium at *b*. Deeper abnormal growth at *c*. × 320.

C.—(Hor.) Semidiagrammatic drawing of active pycnium from 51-day sterile infection. Dead primary paraphyses at *a*, supplanted by secondary paraphyses. Pycnial wall thickened at *b*, *c*. × 320.

How pycniospores brought to the surface of the leaf outside of the pycnium could give rise to binucleate cells at the base of the pycnium is not known with certainty. The evidence at present is limited to a single slide. In this preparation there were two open pycnia extruding spores and there was a continuous mass of exudate on the leaf from one pycnium to the other.

In Plate 11, A, *a*, are represented at high magnification three of the pycniospores within one of the pycnia. In Plate 11, A, *b*, is a similar spore in the exudate outside of the pycnium. In *c* is a spore in the exudate which is pushing out at one end. In *d* the spore has pushed out a short tube at both ends. The spores in *e* and *f* are larger and growing at both ends. In *g* two germinating pycniospores have germ tubes almost meeting at the tips, and *h* shows a similar pair. In *i* and *j* the shape suggests a fusion of two germinating spores, although, of course, other interpretations are possible. Both *i* and *j* occur in the mixed exudate midway between the two pycnia. In *k*, *l*, and *m* are later stages, in which the broadening hyphae are growing toward one or the other of the ostioles.

Some accident before fixation had broken the paraphyses off from one of the two pycnia, leaving the open ostiole with spores issuing from it. In Plate 11, E, is represented the ostiole, *b*, filled with spores, a few of the pycniosporophores, *d*, a bit of the leaf epidermis, *a* to *e*, two broken remnants of paraphyses, *c* and *f*, and several hyphae, *g*, *h*, and *i*, which resemble the younger germinating spores. One of them here, *h*, and one or two others in the next section can be followed directly to the ostiole at *a*. It has not been possible to trace them down into the pycnium.

On this basis, pycniospores can germinate, forming delicate germ tubes, then fuse by pairs, and the fusion cell form a larger, heavier hypha which grows in the liquid of the pycnial exudate to the ostiole. Why, if this can take place, it has not been seen frequently, is not clear. All of this material was fixed by daylight between 8 a. m. and 8 p. m. Perhaps pycniospores grow at night.

Between the pycnium and the edge of the aecium (pl. 12, A) the mycelium is made up of a mixture of gametophytic and sporophytic hyphae. The hyphae run irregularly. No one binucleate hypha can be traced all the way from pycnium to aecium, but several scattered binucleate cells are present. (Pl. 12, A, *c*, *d*, *f*, *g*.) There are no diploid cells at *h*, but two or three sections farther on, at the center of the young aecium, there are several.

The aecium begins in the lower mesophyll tissue of the leaf as a loosely woven snarl of slender intercellular hyphae. In Plate 12, B, is drawn a section through a young aecium from a 13-day infection. It is the youngest of a series formed in quick succession and is located near the margin of the infection. Only the presence of an occasional

EXPLANATORY LEGEND FOR PLATE 11

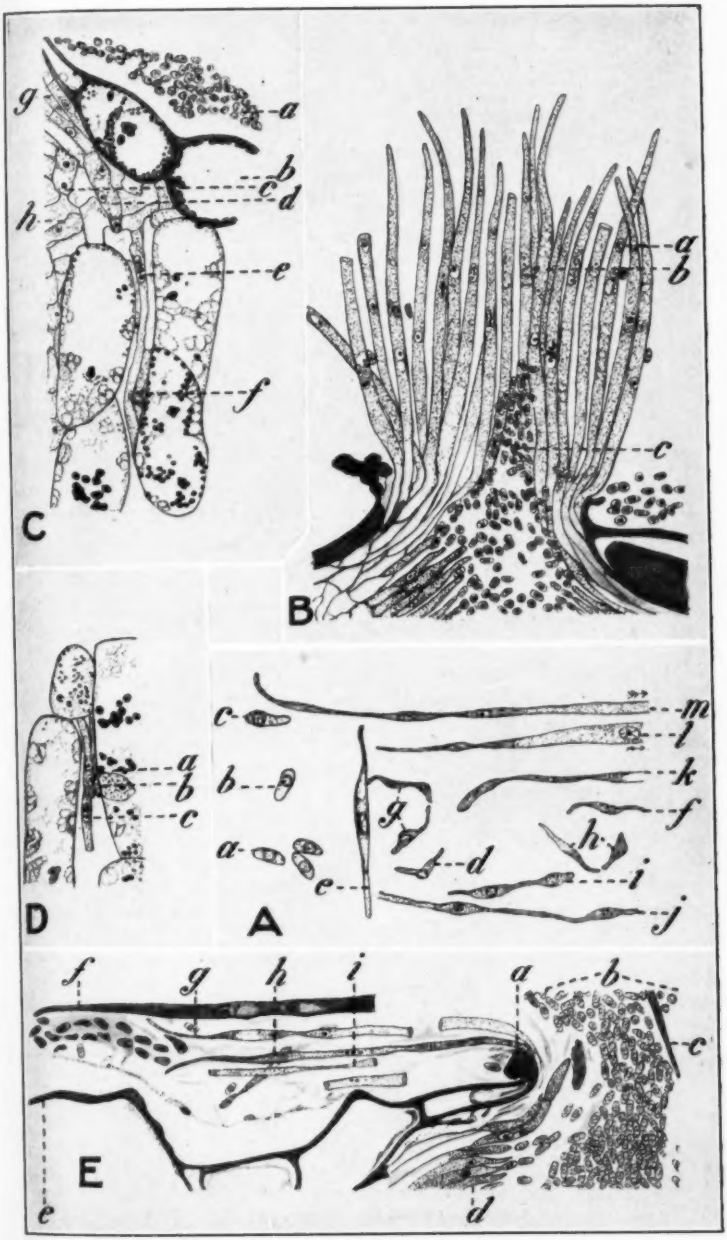
A.—(Trit.) Drawn from a mass of exudate between two pycnia in 6-day material. In *a* and *b*, ungerminated spores. Stages of germination in *c*, *d*, *e*, and *f*. In *g* and *h* are pairs of germinating pycniosporophores. In *i* and *j* two germinating pycniosporophores may have fused. Later stages of growth in *k*, *l*, and *m*. × 1,400.

B.—(Trit.) Same infection as A. Tuft of paraphyses showing nuclear content, *a*, *b*. Spores at *c*. × 730.

C.—(Agr.) Ten-day fertile infection. Margin of pycnium, *h*, with base of paraphyses at *g*, and spores at *a*, above the epidermis, *b*. Binucleate and trinucleate cells in wall of pycnium, *c*, *d*, and in hyphae from it, *e*, *f*. × 640.

D.—(Agr.) Next section to C. Binucleate cells at *a*, *b*, *c*. × 640.

E.—(Trit.) From same slide as A. Leaf epidermis, *a* to *e*. Ostiole at *b*. Broken paraphyses at *f* and *c*. Hyphae in pycnial exudate growing toward ostiole at *g*, *h*, and *i*. × 1,020.



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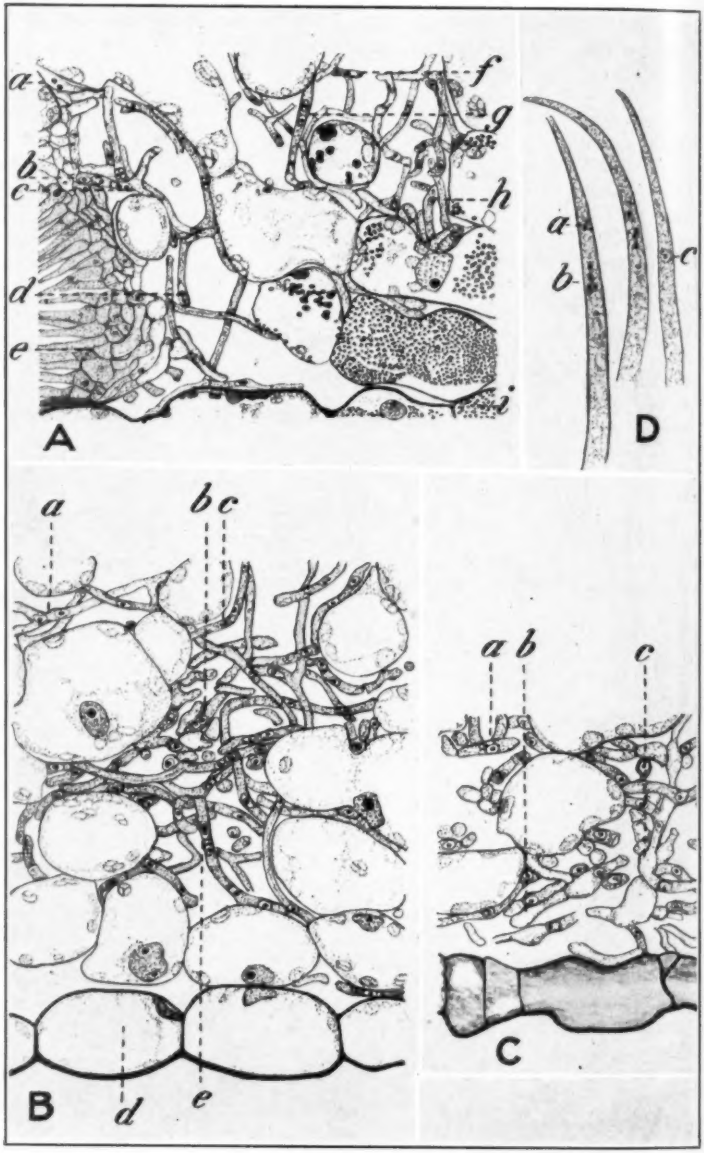

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binucleate cell (pl. 12, B, *a*, *b*, *c*, *e*) distinguishes it from the corresponding stage of development of the sterile aecium. (Pl. 8, B.) There are binucleate cells in the mycelium near by, and older aecia of this same infection show undoubted development toward spore formation.

The next step in development is the appearance of irregular swellings (pl. 12, C), either terminal (*b*) or intercalary (*c*), of the hyphae in the lower half of the aecium. These differ in nowise from those of the corresponding stage in the sterile aecium. (Pl. 8, C.) Here, however, an occasional cell (pl. 12, C, *a*) contains two or more nuclei.

The proportion of diploid cells varies widely in different infections and even in different parts of the same infection. Plate 12, C, and Plate 13, A, are drawn from the same 10-day infection. In Plate 12, C, there is only one binucleate cell. At the base of the pycnium nearest it is a binucleate cell, and there are one or two in the intervening mycelium. In a slightly older aecium of the same infection diploid cells are fairly abundant. (Pl. 13, A, *a*, *b*, *c*, *d*, *e*.) Moreover, one part of an infection can be quite without diploid cells and can remain permanently sterile while the rest progresses to aeciospore formation. The one sterile aecium amidst fertile aecia in Plate 7, B, at *i*, illustrates this.

In Plate 13, A, already referred to, the hyphae by growth and branching have formed a solid nest, in which the differentiation into a lower half (*d* to *f*) composed of large cells with scant content, and an upper half (*a* to *d*) with smaller denser cells, is well marked.

This small solid knot of hyphae increases rapidly in size. Plate 13, B, shows a narrow strip, at right angles to the surface of the leaf, through the center of the aecium. At *d* is the edge of a hypodermal cell of the leaf. From *c* to *d* are rapidly expanding vacuolate cells which will become the sterile outer half of the aecium. From *b* to *c* are smaller denser cells. From this area spores will arise, and the basal area, *a* to *b*, will serve to transfer food to the spore-bearing region. While sporophytic cells are abundant in the area that is to produce spores (pl. 13, B, *f*, *g*, *h*), they are by no means limited to this area. Cells with 2, 3, or even 4 nuclei are scattered from one end of the aecium to the other. (Pl. 13, B, *e*, *f*, *g*, *h*, *i*, *j*, *k*, *l*, *m*.)

Infections of this age (13 days) are particularly favorable for a study of nuclei in areas adjoining the aecia. A little later the mycelial cells will be nearly empty and their nuclei will become indistinct. In Plate 13, C, is drawn a corner of an aecium, *e*, *f*, and mycelium between it and the lower epidermis, *d*. The part of the aecium included is outside of the spore-bearing area, but, as in Plate 13, B, it contains a goodly sprinkling of cells with more than one nucleus (pl. 13, C, *e*, *f*, *g*), and in the mycelium outside there is at least an equal proportion

EXPLANATORY LEGEND FOR PLATE 12

A.—(Agr.) Ten-day fertile infection. Portion of pycnium, *e*, and very young aecium, *h*, and the mycelium between. Lower epidermis at *i*. Binucleate cells at the inner end of the pycnium, *a*, *b*, and in the mycelium, *c*, *d*, *f*, *g*. × 640.

B.—(Agr.) Young aecium from 13-day fertile infection. Binucleate and trinucleate cells at *a*, *b*, *c*, *e*. Lower epidermis at *d*. × 640.

C.—(Agr.) Ten-day fertile infection. Cells of lower half of aecium enlarging, *b*, *c*. Binucleate cell at *a*. × 730.

D.—(Trit.) Fourteen-day infection. Paraphyses fixed a few minutes after mixing pycniospores. Nuclei or nucleuslike bodies at *a*, *b*, *c*. × 1,020.

(pl. 13, C, *a*, *b*, *c*). In Plate 14, A, drawn from the same infection that contains the tissues shown in Plate 13, B and C, is represented the area between two aecia. The edge of one aecium is drawn at *a* and of the second at *e*. In between is to be found an abundance of diploid mycelium. There are mycelial cells with 2, 3, 4, and even 5 nuclei. (Pl. 14, A, *b*, *c*, *d*, *f*, *g*, *h*.) The word "diploid" has been retained here, but whether it is apposite under such circumstances might be questioned.

In Plate 13, D, is a small diagram of an aecium from an infection fixed three days after transferring pycniospores to it, and in Plate 14, B, the upper half of the same aecium is drawn enlarged. Under low power it can not be distinguished from the corresponding stage of devlopment of the sterile aecium (cf. pl. 9, A, and pl. 13, D), but under high power the difference is marked (cf. pl. 9, B, and pl. 14, B). The sterile aecium is composed of gametophytic cells, while the fertile aecium is a mixture of gametophytic and sporophytic cells.

There are now more nuclei in the centrally located sporophytic cells. Earlier (pl. 13, B) 2 nuclei in a cell were common, 3 or 4 in a cell exceptional. There are still binucleate cells, but now 3 and 4 nuclei in a cell are common (pl. 14, B, *d*, *f*, *h*), and the cell at *b* contains 7. As before, there are binucleate cells in the marginal areas (*a*, *c*, *i*, *j*) and in the sterile tissue (*g*). This increase in the number of nuclei per cell would seem to be due to nuclear divisions. No indication of cell fusions has been noted.

There is here the beginning of another change. Near the line of demarcation between the sterile and fertile areas of the aecium (pl. 14, B, *e*) the intercellular spaces are becoming filled with a homogeneous substance, perhaps derived from the breakdown of the walls of the sterile tissue.

The multinucleate cells now elongate, pushing out into the sterile tissue as they grow. Plate 15, A, *f* and *g*, shows an early step in this process, while *b*, *c*, and *e* are somewhat more advanced. Marginal multinucleate cells, as at *a*, sometimes develop later than the central ones, while more remote cells, as at *i*, may take no share in this activity. In this marginal area the cytoplasm of the cells is thin, and many of the nuclei are indistinct.

Material fixed four days after mixing pycniospores of different infections on a leaf shows all stages in the aecia, from the multinucleate cells of Plate 15, A, to young spore chains. In Plate 15, B, is drawn a small group of multinucleate cells slightly older than those above. Here are elongated cells containing 8 or 10 nuclei, with the oldest (pl. 15, B, *c*, *f*) shaping toward cell division. Coming up between the larger cells are smaller ones with 4 or 5 nuclei (*b*, *e*), and still smaller ones with 2 (*a*, *d*).

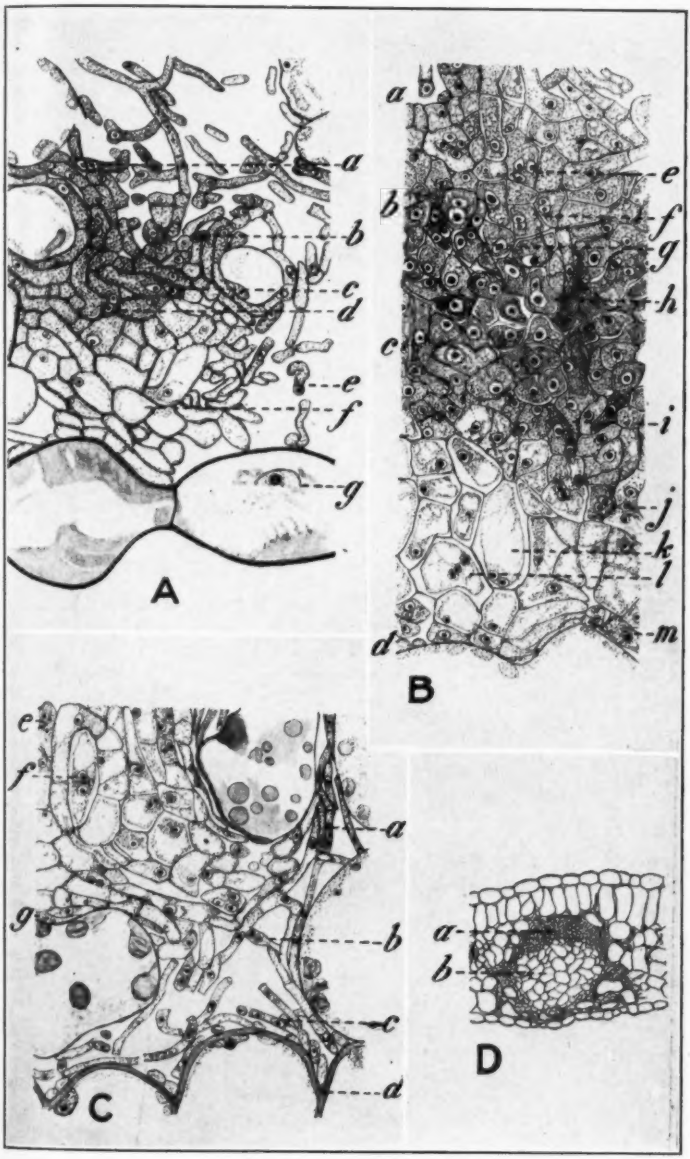
EXPLANATORY LEGEND FOR PLATE 13

A.—(Agr.) Slightly later stage of development of fertile aecium from 10-day infection. Upper half, *a*–*d*, composed of small dense cells; lower half, *d*–*f*, of larger vacuolate cells. Sporophytic cells at *a*, *b*, *c*, *d*, *e*. × 730.

B.—(Agr.) Narrow strip through older fertile aecium from 13-day infection. From *a* to *b* is the basal tissue; *b* to *c*, sporogenous area; *c* to *d*, sterile "space-making" area. Sporophytic cells at *e*, *f*, *g*, *h*, *i*, *j*, *k*, *l*, *m*. × 730.

C.—(Agr.) Thirteen-day fertile infection. The lower corner of an aecium and adjoining mycelium showing presence of cells with 2, 3, or 4 nuclei at *a*, *b*, *c*, *e*, *f*, *g*. Lower epidermis at *d*. × 730.

D.—(Agr.) Diagram of older fertile aecium, fixed 3 days after mixing the spores. Sporogenous tissue at *a* and large empty cells at *b*. × 115.



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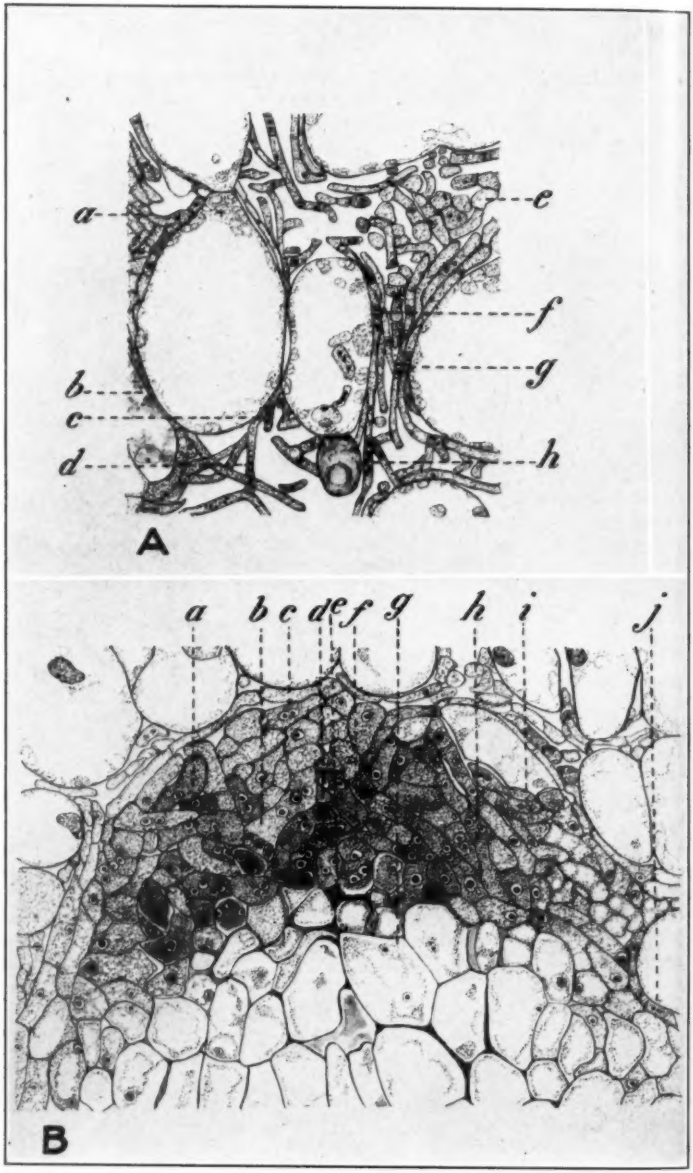

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Notwithstanding the fact that at this stage the tips of these multinucleate cells may contain 5 or 6 nuclei (pl. 15, B, *c, f*), the first cell division usually (not always) cuts off a binucleate cell. Very few terminal cells have been seen with more than 2 nuclei, and later cells are quite uniformly binucleate. In Plate 15, C, there is a young spore chain consisting of a basal cell, *c*, and two young binucleate spore mother cells, *d, e*. Soon after this the older spore mother cells divide, forming the spore and the intercalary cells. In Plate 15, C, *b*, the nuclei have divided, preparatory to cell division, and in Plate 15, D, the division into a large spore, *d*, and a small intercalary cell, *c*, is complete. The number of nuclei in the basal cell decreases as spore formation progresses, but occasionally, even after the young spore chains are well begun, there may still be 3 (pl. 15, C, *a*), 4 (pl. 15, C, *c*), or even 5 (pl. 15, D, *a*).

Plate 15, E, is a diagram of an aecium bearing young spores. The sporophytic growth, *a*, forms a hemispherical mass arching into the sterile area, *b*. In Plate 15, F, the spore chains and adjoining cells of the same aecium are drawn enlarged. The young spore chains here are well organized and the basal cells (pl. 15, F, *b, e*) are regularly binucleate. The inner end of the basal cell may be broad or narrow, squared or irregular. In other rusts, basal cells have been described as arising from the fusion of two cells, with the result that the cell presents a "2-legged" appearance. If such fusions occur in *Puccinia graminis* they are rare. The basal cell at Plate 15, F, *e*, might be so interpreted. Even in this case, however, the possibility is not excluded that it represents the branching of a basal cell rather than the union of two to form one. Of course fusions could occur undetected, since the two "legs" would not necessarily lie within the same section, but it is unlikely that if they occur regularly a few would not be in the plane of the section.

The breakdown of the sterile cells before the advance of the sporophytic tissue, the beginning of which was noted in Plate 14, B, *e*, and which is more evident in the later stage in Plate 15, A, *d*, is now pronounced. (Pl. 15, F, *i*.) A comparison of the three figures on this point suggests that it is not a mere matter of crushing the empty cells. Appearances suggest that the walls are being dissolved.

The spore chains now increase rapidly in both size and number and soon fill the whole interior of the aecium, taking the place of the sterile tissue of the younger aecium. On this account the sterile tissue has often been called "space-making tissue." The diameter of the aecium as a whole increases, as may be seen by comparing the diagrams in Plate 15, E, and Plate 16, A, which are drawn at the same magnification. The basal cells have elongated (pl. 16, A, *a*), and coming from each is a chain of 10 or 12 spores. The older spores are mature and lying loose within the cavity. The whole spore-bearing mass is enveloped in a peridial layer, *b*, derived on the sides from the peripheral spore chains and on the central arch from the terminal cells of the central chains. The expansion of the aecium has placed the leaf epidermis under strain, and the first break has occurred at *c*.

EXPLANATORY LEGEND FOR PLATE 14

A.—(Agr.) From a 13-day fertile infection, showing the edge of one aecium at *a* and of another at *e*, and the mycelium between. Cells with 2, 3, 4, or 5 nuclei at *b, c, d, f, g, h*. × 730.

B.—(Agr.) Portion of Plate 13, D, enlarged. Cells with two or more nuclei at *a, b, c, d, f, g, h, i, j*. Beginning of breakdown of walls of space-making tissue at *e*. × 730.

Plate 16, B, represents the basal cells and adjoining areas of Plate 16, A, enlarged. It is drawn to the same scale as Plate 15, A and F. Comparison shows a marked increase in the number of spore chains. In Plate 15, F, little cells from the base of the aecium (*a*, *d*, *f*, *g*) are elongating and forcing a narrow passage down between the established chains. That spore chains can arise from such beginnings is indicated by basal cells like those in Plate 16, B, *a*, *b*, and *c*, which take their origin from slightly deeper layers of the base of the aecium than the rest. The cells still deeper in the base of the aecium here are so far drained that the cytoplasm is vague and the nuclei ordinarily invisible. Only from younger stages can it be learned that many of these cells belong to the sporophyte generation.

The later history of the pycnium in infections bearing aeciospores differs markedly from that in sterile infections. When fertilization has taken place and aeciospores are developing, the production of pycniospores stops and the pycnial exudate dries. So certain is this that in working with living infections the drying of the epidermis above an infection can be counted on as proof that the diploid phase has begun and that aecia will open on the lower surface in a day or two.

A cytological study of pycnia in infections that have been fertilized shows none of the secondary changes found in sterile infections. The primary paraphyses wither, but no secondary paraphyses form. The ostiole of the pycnium remains narrow and there is comparatively little thickening of the pycnial wall and none of the abnormal basal growth of the pycnium. There may still be old spores within the pycnial cavity, but a study of the tips of the pycniosporophores shows no evidence that new spores are forming. The pycniosporophores soon elongate, blocking the central cavity, and then die.

In Plate 17, A, is drawn a group of paraphyses from an infection several days after fertilization. In the 20-day infection from which this was drawn the aecia are soon to open and many of the pycnia have withered paraphyses. Here, however, the paraphyses are still living. The nuclear content is much the same as it was before fertilization. There are usually two larger, centrally located nuclei, *b*, *c*, and occasionally one (more rarely two) smaller bodies nearer the tip, *a*, which look like nuclei.

In Plate 16, C, is drawn a typical spent pycnium from an old (36 days) fertile infection. With its withered paraphyses, *b*, to which there still adheres a bit of dead, dry exudate, *a*, its narrow ostiole, and its pycniosporophores, *c*, elongated and dying, it forms a marked contrast to the much older but still living and active pycnium of the sterile infection. (Pl. 10, C.)

EXPLANATORY LEGEND FOR PLATE 15

A.—(Trit.) Portion of accium from 12-day fertile infection. Multinucleate cells, *c*, *e*, *f*, pushing out into sterile area. Breakdown of walls of sterile cells at *d*. Other sporophytic cells at *a*, *b*, *g*, *h*, *i*. × 730.

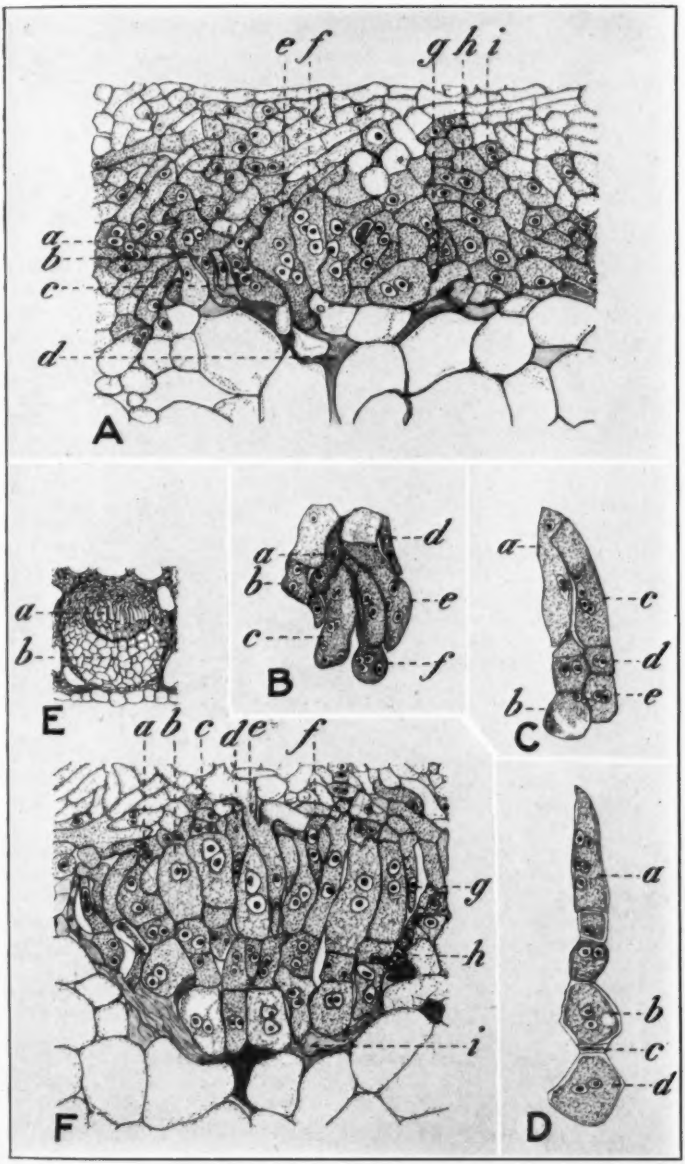
B.—(Agr.) Detail of sporogenous tissue from aecium 4 days after mixing pycniospores. Larger basal cells at *c* and *f*, smaller at *a*, *b*, *d*, *e*. × 730.

C.—(Agr.) Same material as B. Young spore chains showing multinucleate basal cells, *a*, *c*, and spore mother cells at *b*, *d*, *e*. × 730.

D.—(Agr.) Same material as B. Older spore chain, still with multinucleate basal cell, *a*. Spore mother cell at *b*, and spore, *d*, with intercalary cell, *c*. × 730.

E.—(Trit.) Diagram of fertile aecium of 12-day infection, showing young spore chains, *a*, and sterile tissue, *b*. × 115.

F.—(Trit.) Spore chains of E enlarged. Basal cells, *b*, and spore mother cells, binucleate. Younger basal cells at *d*, *f*, *g*, *h*. Possible fusion at *e*. Further breakdown of sterile tissue at *i*. × 730.



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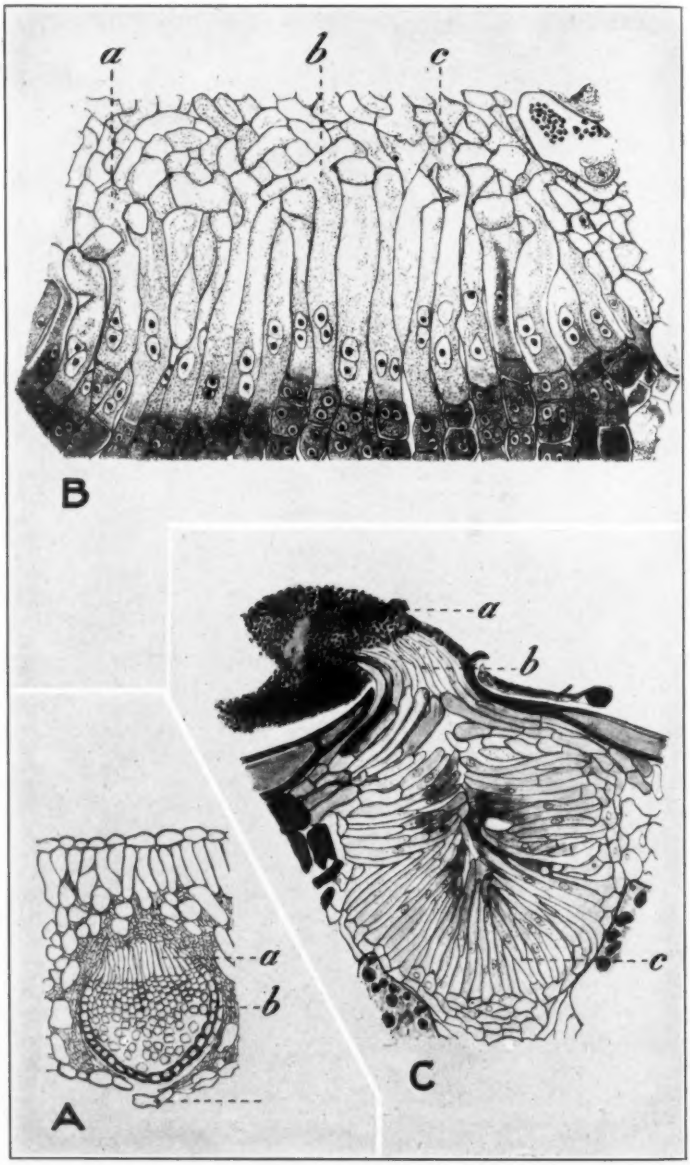

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IRREGULARITIES

The question arises whether *Puccinia graminis* ever reproduces homothallically. Haploid aecia of various ages have been canvassed for evidence. Occasionally an isolated infection bearing several haploid aecia will show a single fertile aecium. A study of the mycelium about its base usually shows the presence of one or more diploid cells, so it may be that here, too, the sporophyte generation arose before the aecium formed. The time, place, and method of its starting have not been traced. Without more information these sporadic fertile aecia in otherwise sterile infections can not be considered unquestionably homothallic.

One or two other irregularities have been found. In one case, two 36-day infections are in contact and their mycelia have interlaced, and both bear open aecia. Among the fertile aecia is one that is seemingly sterile. Under low power it looks like a typical old haploid aecium with large, nearly empty, dying cells. Under high power (pl. 17, B) there is found a central patch of three or four cells whose intervening walls are disappearing. Nuclear divisions have occurred, and the cytoplasm is far richer than in the surrounding cells. The preceding and following sections show only large dying cells like the ones surrounding the patch in the drawing. This seemingly haploid aecium, several weeks old and nearly dead, has suddenly formed by cell fusion the beginning of a sporophyte.

This occurred where two mycelia of opposite sex had interlaced. It is possible that this haploid aecium was composed of hyphae of both mycelia and that the cells which fused belonged to different sexes. Why, on such a basis, the fusion would be so long delayed is not clear; but, at any rate, this too can not be considered unquestionably homothallic.

Another irregularity is depicted in Plate 17, C. In this case two mycelia of the same sex overlapped and both remained sterile for several weeks. Then one of the 14 aecia formed an abnormal sporophytic growth. (Pl. 17, C.) It is too late to trace its origin, but there are several binucleate cells in the margin. (Pl. 17, C, *a*.) Very irregular multinucleate cells occur at the center (*b*, *d*), some of which are dying (*e*). There is an apparent attempt at spore formation, *c* being an abortive spore. It is doubtful whether normal spores could form. Both the fungus and its host are far spent.

DISCUSSION

The germination and entry of the sporidium of *Puccinia graminis* differ from the corresponding stages in the uredinial generations. The urediniospore forms a long germ tube which grows to a stoma and passes through into the substomatal air chamber. The mycelium is intercellular from the beginning. The sporidium pushes out a short beaklike protrusion which pierces the outer epidermal wall directly. Its first life in the host is intracellular.

EXPLANATORY LEGEND FOR PLATE 16

A.—(Trit.) Twelve-day fertile infection. Diagram of a nearly mature fertile aecium with elongated basal cells at *a*, peridium at *b*, and broken epidermis at *c*. × 115.

B.—(Trit.) Portion of A at *a*, enlarged, showing elongated binucleate basal cells, sometimes with irregular bases, *a*, *b*, *c*. × 730.

C.—(Agr.) Old dead pycnium from a fertile 36-day infection. Withered primary paraphyses at *b*, with adherent dead exudate, *a*. Dying or dead elongated pycniosporophores at *c*. × 460.

Perhaps correlated with this is the fact that in the efforts of the fungus to get through the very compact palisade layer of the leaf the intracellular growth there too is more or less mycelial in character. Even the haustoria formed later in mesophyll cells may be made up of more than one cell. It is probable, however, that all of these intracellular growths, whether haustorial in form or intermediate between that and ordinary hyphae, are haustorial in function and serve to extract food from the host. Perhaps here, too, as has been claimed for the uredinial generations of corn rust (*47*), the mycelium absorbs some food directly through hyphae, in addition to what it takes through specialized intracellular organs.

It was hoped at the outset of this work that traces of entry would persist and be detectable in older infections, so that if two or more entries occurred close together and the mycelia became intimately mixed it would still be possible to prove the double or multiple origin. Unfortunately, it is rare to find traces of primary hyphae after the fifth or sixth day. This adds an element of uncertainty in interpreting results. If an infection, supposedly single, and covered to exclude insects, develops fertile aecia, there is always the doubt whether it started as one or as two. Sometimes a careful cytological study resolves the doubt, but sometimes it can not be decided with certainty. In counting infections on the living host plant, a higher percentage develops open aecia than would be expected theoretically. The existence of doubles that look like singles, and trebles that look like doubles, is doubtless a factor in this discrepancy.

In *Puccinia graminis* it would appear that the sporophyte generation does not start in the aecium, nor in the mycelium (at least not commonly), but in the pycnium, and that pycniospores from another and different infection are necessary to its inception. The pycnium can no longer be considered functionless, for it has a vital function, in heterothallic rusts at least. Nor can it be considered a vestigial male organ, for, as Craigie (*19*) has pointed out, half of the pycnia are of one sex and half of the other, although they look alike.

In isolated unisexual infections of *Puccinia graminis* the pycnia continue to produce pycniospores as long as the fungus lives. When the primary paraphyses wither, a second and larger crop of paraphyses is produced. In infection in which fertilization has taken place, on the contrary, the pycnia stop forming spores, the pycniosporophores elongate and block the pycnial cavity, the primary paraphyses wither, no secondary paraphyses form, and the whole pycnium soon dies. The contrasting behavior in the two cases is striking. The pycnium has been assumed to be functionless, but this picture, taken as a whole, is not what would be expected of a functionless organ.

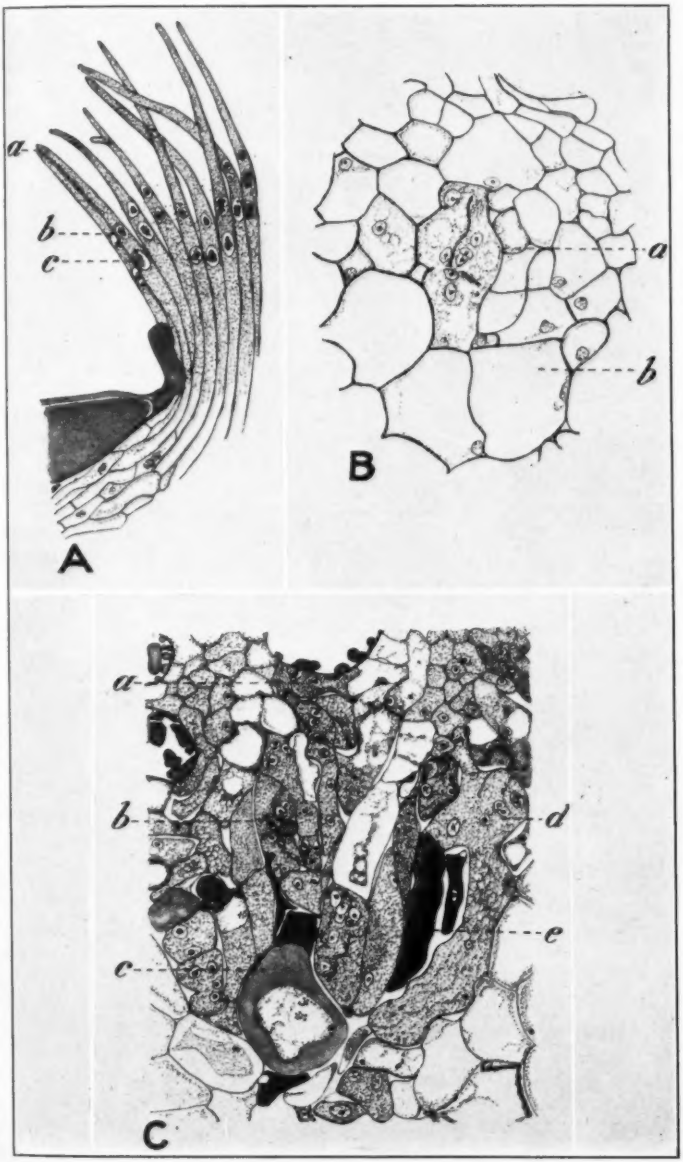
The gametophytic component of the aecium forms regularly, with or without the advent of the sporophyte generation. Evidently no stimulus connected with the presence of diploid cells is necessary for

EXPLANATORY LEGEND FOR PLATE 17

A.—(Agr.) Paraphyses of pycnium of 20-day infection, several days after fertilization. They contain usually two nuclei, *b*, *c*, with sometimes an additional smaller one, *a*. × 730.

B.—(Agr.) Fusion of several cells at *a* in the center of an old haploid aecium of a 36-day fertile infection. Surrounding cells, *b*, nearly dead. × 1,020.

C.—(Agr.) Abnormal young sporophytic growth in one of two old overlapping sterile infections. Binucleate cell at *a*. Multinucleate at *b*, *d*, *e*. Abortive spore at *c*. × 730.



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its formation. This has its parallel in other groups of fungi. Derx (*21*) found haploid sterile perithecia in monosporous cultures of Penicillium. Dodge (*27*) found "sterile bodies characteristic of haplont mycelia" in Neurospora. Mounce (*39*) describes the formation in a few cases of shallow pores on haploid mycelia of *Fomes pinicola*. And Hanna (*32*) found in *Coprinus lagopus* that haploid mycelia can produce imperfect fruit bodies. A few produce spores which are all of the same sex as the parent mycelium.

Just how widespread heterothallism is among rusts is at present a matter of conjecture only. Judging by the rapidly increasing list of heterothallic species in other groups of fungi, it will be found to be fairly common. It has long been noted that in both long-cycle and short-cycle rusts, pycnia appear regularly before the onset of the sporophyte generation. This fact now gains added significance. It may indicate that the pycnium of other rusts is functioning as it does in *Puccinia graminis*. Comparatively few rusts lack the pycnium entirely.

In the long list of rusts that have been studied cytologically, the majority have been described and figured as showing the origin of the sporophyte in the young growing aecium. There the haploid cells of the sporogenous area either fuse two by two (*14*) or the nucleus of one of a pair of cells migrates into the other (*3*). The binucleate cells so formed give rise to the spore chains. In some of the short-cycle rusts the sporophyte originates in a similar fashion in the primary uredinial sorus or in the telial sorus. The following list has been compiled:

Blackman, 1904 (*3*).—*Phragmidium violaceum* Wint.
Christman, 1905 (*14*).—*Phragmidium speciosum* Fr., *Caeoma nitens* S., *Uromyces caladii* Schw.
Blackman and Fraser, 1906 (*4*).—*Uromyces poae* Raben., *Melampsora rostrupi* Wagn., *Puccinia poarum* Niels.
Christman, 1907 (*15*).—*Phragmidium potentillae canadensis* Diet.
Olive, 1908 (*43*).—*Triphragmidium ulmariae* (Schum.) Link, *Gymnoconia interstitialis* (Schlecht.) Lagerh.; *Phragmidium potentillae canadensis* Diet., *Chrysomyxa abietes* (Wallr.) Ung., *Puccinia transformans* E. and E.
Dittschlag, 1910 (*23*).—*Puccinia falcariae* Pers.
Hoffmann, 1912 (*35*).—*Endophyllum sempervive* (Alb. and Schw.) De Bary.
Fromme, 1912 (*29*).—*Melampsora lini* (Pers.) Desm.
Fromme, 1914 (*30*).—*Puccinia claytoniata* Peck., *P. violae* (Schum.) DC., *P. angustata* Peck, *P. hydrostyles* (Link) Cke., *P. eatoniae* Arthur, *Uromyces caladii* Farlow.
Colley, 1918 (*16*).—*Cronartium ribicola* F. de Waldh.
Adams, 1920 (*1*).—*Peridermium comptoniae* Orton and Adams, *P. pyriforme* (Peck) Hedgc. and Long, *P. acicolum* Underw. and Earle, *P. peckii* Thüm., *P. balsameum* Peck.
Thurston, 1923 (*57*).—*Gymnosporangium bermudianum* Farlow.
Lindfors, 1924 (*38*).—*Melampsora reticulata* Blytt., *Coleosporium euphrasiae* (Schum.) Wint., *Uromyces acetosae* Schrot., *Puccinia tragopogi* (Pers.) Cda., *Triphragmidium ulmariae* (Schum.) Link, *Trachyspora alchemilliae* (Pers.) Fuck., *Puccinia morthieri* Koern., *Chrysomyxa abietes* (Wallr.) Ung.
Petry and Hutton, 1924 (*46*).—*Dicaeoma distichlidis* (Ellis and Ev.) Kuntze.
Walker, 1927 (*60*).—*Puccinia cryptotaeniae* Peck, *P. xanthii* Schw., *P. fusca* (Pers.) Relb.

The above list is not complete but suffices to show the range. These rusts would appear at first sight to be homothallic. Even here, however, the possibility is not excluded that in some cases the haploid tissue in the young sorus includes (+) and (−) hyphae of separate origin and that a sorus without both elements would remain sterile.

Dodge (*24*, *25*) found a situation in the short-cycle orange rust of Rubus (*Caeoma nitens* Burrill) that is suggestive. Some strains of the rust produce no pycnia, there are no cell fusions in the base of the aecium, the aeciospores formed are for the most part uninucleate, and the aeciospores germinate by forming 2-cell promycelia with two uninucleate sporidia. Other strains of the short-cycle rust produce pycnia, cell fusions occur in the base of the aecium, and binucleate aeciospores are produced which on germination produce (probably without either nuclear fusion or reduction) 4-cell promycelia with four sporidia. Heterothallism and the agency of the pycnium in fertilization here are probable, but the details of the process must differ from those in *Puccinia graminis*, for fusions are delayed until the young aecium has formed.

Although the majority of rusts that have been studied cytologically show cell fusions in the aecium or in the corresponding sorus in short-cycle forms without aecia, instances are not lacking in the literature in which binucleate hyphae are present at an earlier stage. Blackman and Fraser (*4*, *p. 40*) saw paired nuclei in the aecium in *Puccinia poarum* Niels. "before the differentiation of the fertile layer, or in cells below that layer after its differentiation." Olive (*45*) saw mixed perennial gametophytic and sporophytic mycelia in several species and found that in *Puccinia podophylli* Schw. aecia may arise from preexisting binucleate hyphae. Lindfors (*38*, *p. 16*) studied the development of aecia of *Puccinia tragopogi* (Pers.) Cda. in very young leaves and found that—

> Es konnte konstatiert werden, dass die Zellen in fertilen Teil in weitem Masse mehrkernig waren, und dass dieser Zustand schon in denjenigen Hyphen herrschte, die sich von aussen an die Äzidienanlage anlegten.

In short-cycle rusts the binucleate condition often arises before the formation of basal cells in the sorus and even in the mycelium outside. This has been reported in *Puccinia malvacearum* Mont. by Blackman and Fraser (*4*), and of the microforms *Uromyces scillarum* Wint. and *Puccinia adoxae* DC. they say (*4*, *p. 43*): "The general vegetative mycelium showed conjugate nuclei." In *Uromyces ficariae* Lév. (*4*, *p. 43*), another microform, the general mycelium was uninucleate but "the mass of mycelial hyphae round the teleutospore sorus, as well as those directly connected with teleutospore formation, appear to have conjugate nuclei." Lindfors (*38*) found widespread binucleate mycelium in *Puccinia albulensis* Magn., *P. epilobii* DC., *P. gigantea* Karst., *P. holboelli* (Hornem.) Rostr., *P. saxifragae* Schlecht., and *Uromyces solidaginis*. Walker (*60*) found a similar condition in *Puccinia asteris* Schw. and *P. cryptotaeniae* Peck.

The probability is that some of these short-cycle rusts will be proved to be heterothallic. This can not be taken for granted, however, for there is an alternative. Lindfors (*38*) finds that in *Puccinia arenariae* (Schum.) Wint., a lepto-form, the teliospore gives rise to a 2-cell promycelium with two binucleate sporidia. The mycelium is binucleate and produces teliospores directly without cell fusions. This is certainly a homothallic species. Further study of these short-cycle rusts is needed to show whether the binucleate mycelium arises in connection with the pycnium (heterothallism) or in the promycelium (homothallism).

The evidence at hand as to the mode of origin of the sporophyte generation in *Puccinia graminis* is suggestive but not conclusive.

Without further supporting evidence, neither the fusion of two pycniospores nor the fusion of a pycniospore with a paraphysis can be considered proved. Neither would be without parallel in other fungi, for in the basidiomycetes it is believed that binucleate mycelium can arise by the fusion of two oidia, by the fusion of an oidium with a hypha, or by the fusion of two hyphae.

The latter possibility should be considered. When (+) and (−) mycelia interlace, it may be that the sporophyte can arise directly by the fusion of (+) and (−) mycelial cells. A careful search has not disclosed this in *Puccinia graminis* but it may occur none the less. It would be easily overlooked. Other workers on rusts have assumed that a fusion of vegetative cells may take place, and its occurrence in rusts is made more probable by the fact that it is known to occur in some smuts (*33*) and would appear to be of general occurrence in basidiomycetes.

Still another possibility should be considered. When two or more mycelia interlace, it is at least theoretically possible that (+) and (−) hyphae should contribute to the formation of the same pycnium and that both elements should form spores within the same pycnial cavity. There is no way in which this can be either proved or disproved, as (+) and (−) elements look alike. In such a case fertilization might occur within the pycnium without the introduction of spores from without.

In the aecium of *Puccinia graminis* the sporogenous cells are regularly multinucleate, and this condition is brought about, so far as noted, by rapid nuclear divisions. Multinucleate cells in the fertile layer of the young aecium have been reported in other rusts, but they have usually been ascribed to multiple fusions. Several workers have recorded the existence of an occasional trinucleate basal cell which gives rise to a chain of trinucleate spores (*3*, *4*, *43*, *23*, *35*, *29*, *30*, *16*, *38*). Basal cells with more than three nuclei also occur (*16*, *60*, *38*, *57*, *43*, *44*), but have usually been regarded as of exceptional or unusual occurrence. Colley (*16*, *p. 652*) noted, however, that in *Cronartium ribicola* basal cells with more than two nuclei often give rise to binucleate spores, and he states:

> Emphasis should perhaps also be placed on the constant and normal occurrence of multinucleate cells at the base of the æcium, * * *, by suggesting that in deep-seated æcia of caulicolous Peridermia the æciospore chains may be found to arise more often from these placentalike cells than from basal cells arising from the fusion of only two cells in the fertile layer.

Olive (*44*) states:

> Further, a large proportion of the 50 species of æcidium cups under investigation have been found to show a multinucleated stage in their development.

The discovery by Craigie of heterothallism in *Puccinia graminis* suggests that two physiologic forms of the rust growing together on a barberry might hybridize there and that new forms of the rust might result. This would complicate the question of breeding for rust resistance and give added impetus to the work of barberry eradication. The hybridizing of physiologic forms of smuts under controlled conditions has already been achieved (*36*, *31*), and interspecific hybrids have been grown between two smuts (*22*) and between two ascomycetes (*28*).

New forms of the rust also may be found to arise by mutation, for mutations have occurred in the cultures of many fungi (*55*, *58*, *33*, *41*,

37, *40*, *7*, and perhaps *56*). One mutation in rusts has already been discovered by Newton and Johnson (*42*) in *Puccinia graminis*. It is possible that the teratological growth of the pycnia found in a single lot of material of *P. graminis* (pl. 10, B) is due to a mutation. It would be impossible to prove it, however, for it was discovered under the microscope after the material had been fixed.

In several of the mutations cited above, the mutants showed altered sexual behavior. The possibility should be kept in mind that some of the unexplained cases in *Puccinia graminis* in which a single fertile aecium appeared in a unisexual infection may be due to a localized mutation in the mycelium followed by a fusion between the mutant and the parent mycelium.

There is no positive evidence at present of more than two sexual groups in *Puccinia graminis*, but in so large and varied a species, with its numerous physiologic forms, it would not be surprising if other sexual groups were discovered.

SUMMARY

The sporidium of *Puccinia graminis* on a barberry leaf germinates, pierces the outer wall, and enters the epidermal cell, forming there a primary hypha of four to six cells.

From each cell of this primary hypha a branch grows down to the subepidermal area and develops into haploid mycelium. Intercellular hyphae are vigorous and shapely. A few intracellular hyphae form, but they are stunted, misshapen, and short-lived.

Several entries may occur close together, and the mycelia may interlace. No unmistakable case of fusion of vegetative cells has been noted. Young interlaced mycelia remain haploid.

The primary hypha degenerates rapidly and disappears.

Pycnial development begins on the fourth day. Hyphae grow up between palisade cells and form a mat between the epidermal and palisade layers. Branches converge at a common point. Large central upright hyphae lift the epidermis and serve as buffers against its pressure. The pycnium soon becomes organized into an outer wall from which slender-tipped pycniosporophores grow into the central cavity. Each pycniosporophore gives rise to a succession of spores. From around the upper edge of the wall, paraphyses grow in, meet, and turn outward, piercing the epidermis and forming a brush of tapering, stiff-looking hyphae outside of the epidermis. Each paraphysis contains 1, 2, or 3 nuclei.

An isolated unisexual infection produces from 3 or 4 to 2 dozen or more haploid aecia. These attain considerable size, undergo the first differentiation into areas, then become impoverished and die.

Pycnia in sterile infections remain active as long as the host tissues can supply food. When the primary paraphyses wither, secondary paraphyses are formed. Spores continue to be formed in abundance, and a drop of pycnial exudate is maintained on the surface of the leaf.

After pycniospores of one sex have been transferred to an infection of opposite sex, small granules which may be nuclei of pycniospores are found in the paraphyses. Later, cells with two or three nuclei are found in the wall of the pycnium and in hyphae leading from it.

Between a pycnium and a young fertile aecium are many haploid and a few diploid hyphae. Between the successively formed aecia

of a fertile infection there are sporophytic cells with two to four nuclei. Beyond the youngest aecium in the marginal mycelium is an occasional sporophytic hypha.

The gametophytic component of the aecium forms as in the sterile infection, but scattered throughout it from its very inception are sporophytic cells. These cells may contain 2, 3, or 4 nuclei.

Three days after pycniospores were transferred to an infection aecia have attained considerable size and the sporogenous area is well defined. The number of nuclei in the sporophytic cells in this region increases, apparently by nuclear divisions. Cells with three to six nuclei are common.

On the fourth day after fertilization these multinucleate cells push down into the sterile "space-making" tissue of the aecium to produce spore chains. Just before the first division a multinucleate cell contains 8 or 10 nuclei. These extra nuclei are utilized in forming the binucleate spore-mother cells so that the number in the basal cell steadily decreases. In well-established spore chains basal cells are regularly binucleate.

Soon after the advent of the sporophytic generation in an infection the pycnia stop forming spores and the pycniosporophores elongate, blocking the pycnial cavity. The paraphyses wither and no secondary paraphyses form. Pycnia of fertile infections die while the rest of the infection is still living and active.

A few cases of irregular and often abortive development in old haploid aecia have been noted. Their origin and nature are not fully known but may be homothallic in character.

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THE ANATOMY OF EUPHORBIA INTISY[1]

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INTRODUCTION

Among the many peculiar plants that characterize the arid region of southern Madagascar, one of the most interesting is *Euphorbia intisy*.[2] Not only does this plant present several characteristics that make it worthy of considerable study by the theoretical botanist, but its past performance as a rubber-producing plant makes it seem possible that it may prove adaptable to American conditions.

Euphorbia intisy was discovered June 7, 1891, at Tsivory, Madagascar (*8, p. 107–109*).[3] Commercial exploitation followed at once, so that the annual rubber exportation of Madagascar rose from less than 50,000 pounds to almost a million pounds. The ruthless exploitation, however, soon showed its effect, for within a few years intisy had become so rare that Prudhomme (*10*) in 1899, even before the plant had been named botanically (*4*), reported that where intisy had formerly been very common it was already difficult to find specimens.

The manner of obtaining intisy rubber is very simple and is one of the factors that have led to the commercial disappearance of this plant. The latex, which exudes from any cut, coagulates in the air without further treatment. Hence all that the native has to do is to make cuts in the stem, return after a few hours or a few days and pull off bands of rubber of very high quality, which he wraps into balls.

The yield of rubber from stems less than 3 cm. in diameter is so slight that it does not pay to tap them. Consequently, at the present time, because of the disappearance of the large trees, one finds tapping scars only at the collar, the most favored tapping position. (Fig. 1.) It is reported, however (*9*), that during the time of its great exploitation spiral cuts were made all the way up the tree from the ground to branches as small as 2 or 3 cm. in diameter, 15 pounds or more of rubber being obtained at one tapping from a single tree. In many cases the first tapping was so severe that the tree died outright. Trees subjected to less severe tapping in some cases withstood several such onslaughts, but still the weakening—both structural and physiological—incident upon careless cutting, always brought about the death of the tree. From what is known about tapping other rubber plants and about the stem anatomy of this plant, intisy would almost surely be able to live for many years if careful tapping methods were employed.

That this valuable plant occurs throughout a large part of southern Madagascar, that it does not occur in pure stands, and that it requires

[1] Received for publication Oct. 10, 1929; issued April, 1930.

[2] This plant is variously known throughout the region where it grows as intisy, inty, herokazo, herobay, erobahy, herotra, caoutchouc sans feuilles, caoutchouc du sud, caoutchouc Antandroy, kokomy, and pira Mahafaly. The rubber from it is referred to in commerce as kilwa, niggers, or intisy.

[3] Reference is made by number (italic) to "Literature cited," p. 625.

a number of years before it reaches a size sufficient to tap for rubber, have combined to keep the species alive, even though as early as 1906 it was reported that intisy rubber was becoming unobtainable on the market, in spite of its valuable qualities which made it greatly desired in the automobile industry (*2, p. 231*).

FIGURE 1.—General aspect of the root system of *Euphorbia intisy* showing the hydrorhizas and the absorbing roots. Note also the tapping scar at the collar. × ¼

A small number of living specimens that reached France during the first five years of this century made possible a few anatomical descriptions, but intisy proved unadapted to life in French greenhouses. In 1928 it was believed by the authorities on Madagascar plants that *Euphorbia intisy* did not exist except in Madagascar; also, it was feared that the species had become extinct in Madagascar.[4]

One of the chief results of the Humbert-Swingle Madagascar plant-exploration expedition of 1928 [5] was the finding of *Euphorbia intisy* at several places in southern Madagascar and the bringing back to Tananarive, Marseille, Algiers, and Washington of living specimens of this plant. The description that follows is based upon material collected by the writer at Ranohasy, Ambondro, and Behara, Madagascar, in August and September, 1928, and upon material obtained in Washington from the living plants brought back from Behara. In the description the writer has also drawn upon the results of the French investigators who studied this plant.

CLASSIFICATION

Euphorbia intisy (*Euphorbia intisy* Drake), according to Denis (*3*), belongs to the subsection Tirucalli of the section Euphorbium. This subsection includes a number of tropical xerophytic trees and shrubs in which the process of photosynthesis is largely or even completely taken over by the stems, which in most cases are fleshy.

The following floral description is chiefly from Drake (*4*) and Fron (*6*):

Inflorescences are deep red, produced in summer (December to February), borne in short, fleshy terminal cymes with most of the flowers abortive. Flower-

[4] A type distributed as *Euphorbia intisy* var. *mainty* Poisson had been found to be only a type of *E. laro* Drake.

[5] Sponsored by the University of Algiers, the Arnold Arboretum of Harvard University, and the United States Department of Agriculture.

ing is rare and seems entirely confined to old plants. The sexes are separate, but it is still unknown (1930) whether intisy is monoecious or dioecious. Cyathia urceolate with five disciform glands; stamens in five groups of three or four each, arranged with articulate peduncle near the anther; ovary usually bilocular; styles bifid; capsule spherical, slightly flattened, 2 to 3 cm. in diameter; seeds hemispherical, smooth, brown, and ripening about three months after flowering and quickly losing their germinative power.

VEGETATIVE CHARACTERS

The writer has not seen either flowers or fruits of *Euphorbia intisy*, but in the field it is relatively easy to distinguish this plant from the other arborescent Euphorbias which make up the "intisy brush." Its chief characteristics are as follows: (1) Unlike any similar form,

FIGURE 2.—The largest known plant of *Euphorbia intisy* now alive. Photograph taken in the garden of the Chief Administrator, Ambondro, Madagascar, September 8, 1928

the branches are ligneous up to within a few millimeters of the growing point; (2) the youngest branches are of much smaller diameter than those of any similar Euphorbia; (3) the latex is not sticky, but when a bit of it is rubbed in the hand it soon coagulates and seems to contain nothing but pure rubber; and (4) the root system is unique. (See p. 620.)

Intisy is a shrub or small tree, reported to reach a height of 7 meters and a stem diameter of 25 cm. However, no trees of this size are known to exist to-day, the largest that the writer saw being approximately 4 meters high. (Fig. 2.) The stem is upright and frequently branched at the base, but it does not throw up suckers from below the surface of the ground even after the top has been entirely cut away—a point which has doubtless been of considerable importance in the economic disappearance of the plant. The general appearance

of the tree is usually unimposing and straggling. The young stems, however, are dichotomously branched and present a regular, graceful appearance, resembling mistletoe. The stem remains greenish and smooth for many years, the epidermis being covered only by a thick waxy covering. After many years a cork cambium is formed (in the hypoderm or deeper), after which the stem presents a scaly, brown appearance, due to the bark formed by this phellogen.

THE STEM

Intisy is virtually leafless, although in a rapidly growing state it has reduced sessile leaves, lanceolate or spatulate, narrowed at the base, 5 to 15 mm. in length and 1 to 2 mm. in width. Costantin and Gallaud (*1*) reported that the leaves were 30 by 2 mm., and Fron (*6*), 10 by 4 or 5 mm. Fron reported that the lower surface of the leaf was covered with multicellular, clavate hairs, but all leaves and growing points examined by the writer were found to be entirely glabrous. Stomata are sparse on both surfaces. A well-developed cuticle is present and the leaf has a glaucous appearance. The leaves and growing points are more or less red, the reddening being the more pronounced on shoots developed under cool conditions. Occupying the position of the stipules, which are wanting, a gland lies on either side of the leaf at its insertion. In the greenhouse, even under very humid conditions, the leaves fall in a few days, so that a few centimeters behind the growing point no leaves are displayed.

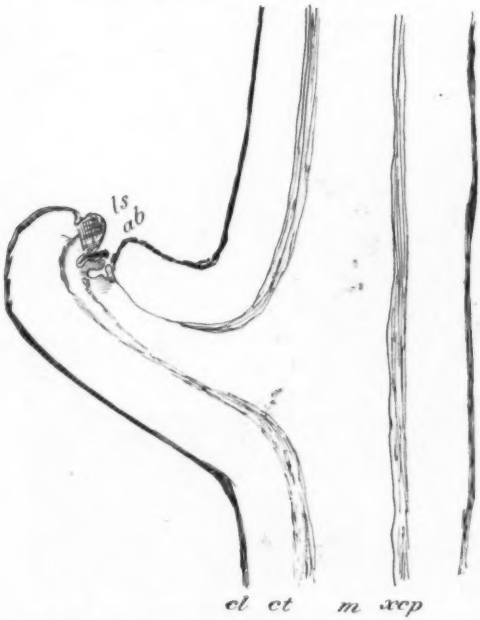

FIGURE 3.—Camera-lucida drawing, showing peculiar type of nodes displayed by certain plants of *Euphorbia intisy*: *p*, Phloem region; *ls*, leaf scar; *ab*, axillary bud; *cl*, cuticle; *ct*, cortex; *m*, pith; *x*, xylem; *c*, cambium. × 15

The usual behavior is for a terminal bud to form after a few centimeters of growth. From this terminal, three or four branches (rarely one, two, or five) arise. Only occasionally do side buds grow out, most branching being of the dichotomous type described.

Some irregularity in phyllotaxy is displayed, but usually it is immediately obvious that the plant is alternate-leaved, having a 2/5 arrangement. Each leaf receives from the stem a single bundle running the entire length of the leaf and giving off numerous lateral branches, which anastomose freely.

Some of the plants show a very peculiar type of emergence in connection with most of the nodes. As shown in Figure 3, the bud with its subtending leaf is borne on a projecting shoulder, which, while superficially resembling a short lateral branch, is not derived from the lateral bud, but is entirely the product of the stem. This unusual structure is found on approximately 30 per cent of the growing plants and on some of the preserved material obtained at Behara. It was not noted in the field and apparently was never observed by any of the early investigators who studied intisy, evidently not being present on the few plants previously available for study. Possibly this feature is of specific rank, though in other vegetative characters the plants seem to be like the ones that do not show this peculiar structure. Also, it is possible that the plants showing this structure are of the opposite sex from the others. At present, however, in the absence of flowers, such suggestions are little more than speculations.

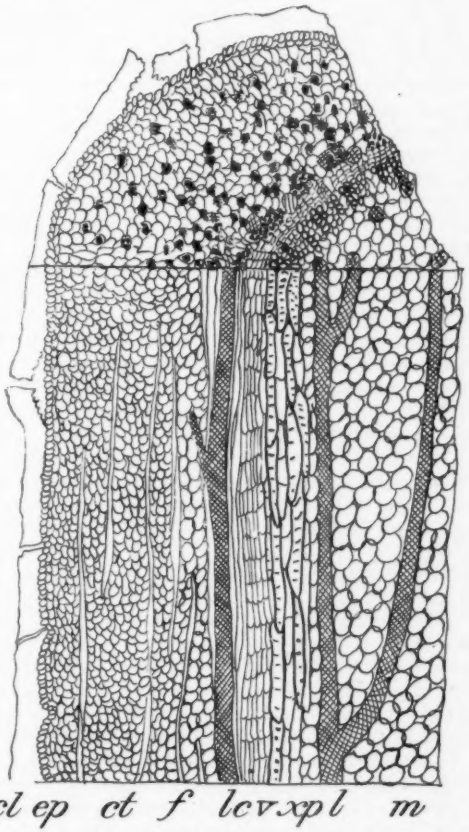

FIGURE 4.—Longitudinal and transverse section through young stem of *Euphorbia intisy*, just behind growing point, taken when dormant: *v*, Vessels; *xp*, xylem parenchyma; *m*, pith; *cl*, cuticle; *c*, cambium; *ep*, epidermis, *ct*, cortex; *f*, fibers; *l*, latex cells. ×80

Figure 4 shows in both longitudinal and transverse section the aspect of the young stem about 2 cm. behind the tip of a dormant branch. The outside is covered by a thick cuticle, and a hypoderm lies just beneath the epidermis. There is no evidence of a cork cambium, this being formed only after many years. Inside the hypoderm lies the broad cortical parenchyma, made up of palisade cells containing much chlorophyll and frequent starch grains. Toward the exterior the cells fit together tightly, so that intercellular spaces occur only among the innermost layers of the cortex. Scattered throughout this entire region lie numerous longitudinal fibers, isolated or in small groups.

A few latex cells are scattered through the cortex. However, most of the latex cells lie just within this region, and the fibers likewise are found most abundantly here in the phloem, occurring in groups of a dozen or more and almost completely setting off the cortical parenchyma from the phloem.

This arrangement of the fibers, radiating from groups (probably of pericyclic origin) lying next to the phloem, is very striking; according to Costantin and Gallaud (*1*), it is shown by no Euphorbias except the Madagascar representatives of the subsection Tirucalli. Moreover, the long fibers, which are either straight or branched, are characterized by not being compressed as are the cortical fibers of most Euphorbias. These fibers are made up of several layers of cellulose, the lumina becoming completely filled at a relatively young stage. Even in their later stages of growth, they remain almost entirely of cellulose. Treatment with hydrochloric acid and phloroglucin shows that the fibers contain but very little lignin, for they color but a faint pink.

The phloem is only slightly developed and appears almost identical with the cambium.

Within the cambium lies the well-developed xylem ring that characterizes the stem of intisy. This is made up of tracheids and vessels in the process of differentiation, interrupted tangentially by the vascular rays and radially by the xylem parenchyma. A large pith, made up of loosely fitting chlorophyllaceous cells larger than those of the cortex, occupies the center of the stem. There are no medullary fibers, but latex cells similar to those found outside the phloem run through the pith.

The latex cells have not been thoroughly investigated, but in all respects (except in the high rubber content of the latex) they seem to be similar to the latex cells of other members of the genus Euphorbia described by Gaucher (*7*). These latex cells consist of very long tubes without cross walls, branched but without anastomoses. The tubes have cellulose walls, evenly thickened throughout. Presumably in intisy also the tubes arise as individual cells in the embryo and continue to grow through the surrounding tissues, generally perpendicularly, throughout the life of the plant. They do not form lysigenously or by the linking up of individual cells, but only in the manner described, like the growth of fungus hyphae. Apparently latex cells never form anew either in wound tissue or from the cambium, though this point does not seem to have been thoroughly investigated.

In cross sections of unstained tissues the latex cells are sometimes difficult to distinguish from the fibers, but because of the fatty nature of the rubber which they contain, these cells stain red with Sudan III.

THE ROOT

The root system of *Euphorbia intisy* is apparently unlike that of any other plant. Figure 1 illustrates the general appearance of a part of the root system, showing the absorbing roots and the swollen, water-storage hydriarhizas;[6] these, alternating with the short, constricted segments, give the entire root somewhat the appearance of a string of short, thick, link sausages. Although water-storage roots

[6] "Hydriarhiza" is used here for the first time. From hydria, a water jug, + rhiza, a root, emphasizing the localized nature of the water-storage tissues.

are well known—e. g., *E. tuberosa* L.—the writer knows of none characterized by localized swellings such as these hydriarhizas of *E. intisy*.

Dubard and Viguier (*5*) have described the primary structure of the root, showing that the young tips of the hydriarhizas (erroneously called tubercules) and those of the absorbing roots are essentially identical and that no unusual anatomical characters are displayed in these portions.

Figure 5 shows the appearance of a portion of the root lying between two hydriarhizas. This root had undergone considerable secondary thickening, but of the usual sort and not of the anomalous type that characterizes the hydriarhizas themselves. Except for this radial thickening, the root structure at this point presents an aspect but slightly different from that of the stem shown in Figure 4. In the root, however, the cork cambium, which arises in the pericycle, has cut off considerable phelloderm. Owing to the expansion and occasional appearance of cross walls in the radial plane in this tissue, the serial arrangement of the phelloderm is soon lost, so that what is actually secondary tissue, derived from the cork cambium, appears almost identical with the primary cortex of the stem. It is not possible at this stage to locate the boundary between this phelloderm and the phloem or to tell which of the cells were part of the original pericycle.

The latex cells are slightly more numerous in the root than in the youngest stems, but in the root they all lie outside the cambium, none being present in the pithlike axial tissue.

Figure 6, which is drawn on the same scale as Figure 5, shows a section through one of the hydriarhizas on the same root, 2 cm. from the place where the transverse section shown in Figure 5 was made. The most striking point brought out here is the enormous enlargement that the cells of the axial tissue and the xylem parenchyma have undergone. In many cases these tissues have increased in volume a thousand times, yet apparently there has been no division of any of the cells lying inside the cambium. The walls of these aquiferous cells, which are stretched very thin, do not lignify.

Scattered through this region occur islands of xylem which have been forcibly pulled apart by the swelling of the intervening parenchyma cells. These constitute irregularly oriented strands, which, however, run in a general vertical direction.

Outside the cambium there has been little alteration in cell size, the cells having responded to the centrifugally exerted pressure by forming radial cross walls in the phelloderm and phellogen. The cells of the cork itself, being inelastic, are ruptured by this increase in girth, and characteristic cracks appear. (Fig. 1.) Apparently there is no increase in the number of fibers, for a given area in Figure 6 shows fewer fibers than a corresponding one in Figure 5. Also, as indicated in Figure 6, the fibers have been more or less displaced by the pressure exerted from within the cambium.

Gaucher (*7*) emphasized the fact that one of the characteristics of the Euphorbias is that the latex cells do not grow into the phloem ("liber"), a point which seems to hold with intisy for the stem. Undoubtedly, however, as Figures 5 and 6 show, latex cells do grow into the older phloem of the root, though not into the younger.

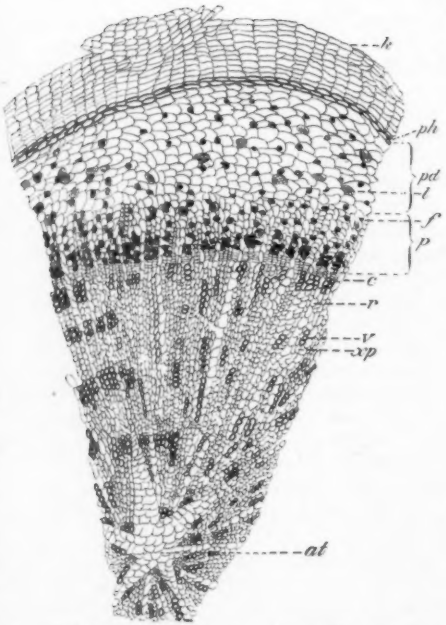

FIGURE 5.—Transverse section through unswollen portion of root of *Euphorbia intisy:* *c*, Cambium; *k*, cork; *ph*, phellogen; *pd*, phelloderm region; *l*, latex cells; *f*, fibers; *p*, phloem region; *r*, xylem rays; *v*, vessels; *xp*, xylem parenchyma; *at*, axial tissue. × 25

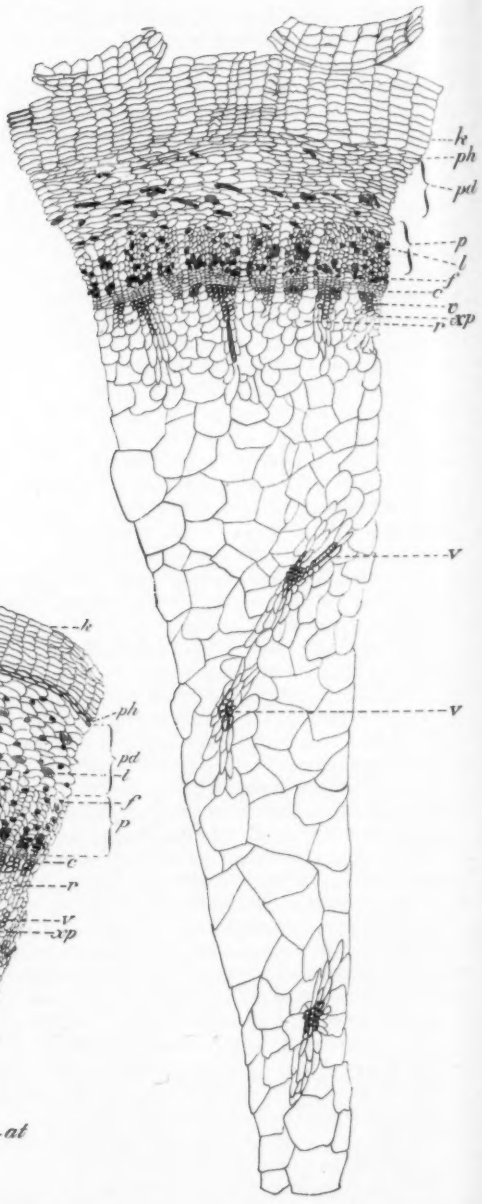

FIGURE 6.—Transverse section through hydriarhiza of *Euphorbia intisy:* For explanation of letters see Figure 5. × 25

Figure 7 shows the appearance of a dried root. The transverse plates so strongly indicated are the collapsed aquiferous tissue.

As Dubard and Viguier (*5*) pointed out, the anomalous swelling that leads to the production of these characteristic hydriarhizas commences early, usually with the appearance of secondary growth, the axial tissue undergoing considerable distension. This enlargement soon reaches into the ring of secondary xylem, the primary rays swelling, chiefly in the tangential plane. Frequently one of the six primary bundles fails to lignify, the cells of the bundle undergoing the same fate as those of the primary rays.

Next, all the secondary xylem rays enlarge, displacing the rows of vessels, and finally almost complete disruption is brought about when the xylem parenchyma cells, lying between the individual vessels, likewise enlarge. Except for the widely separated strands of vascular bundles that in cross section appear as scattered islands, the tissue appears almost homogeneous, and at this stage it is impossible to tell whether a given cell has been derived from an aborted primary vascular bundle, from axial tissue, from a primary or secondary ray, or from xylem parenchyma.

The aquiferous tissue is almost entirely water. The hydriarhizas are frequently consumed by the natives of Madagascar as a source of water, and the writer found that the slight taste of the watery pulp detracts but little from the value of these hydriarhizas to the thirsty traveler.

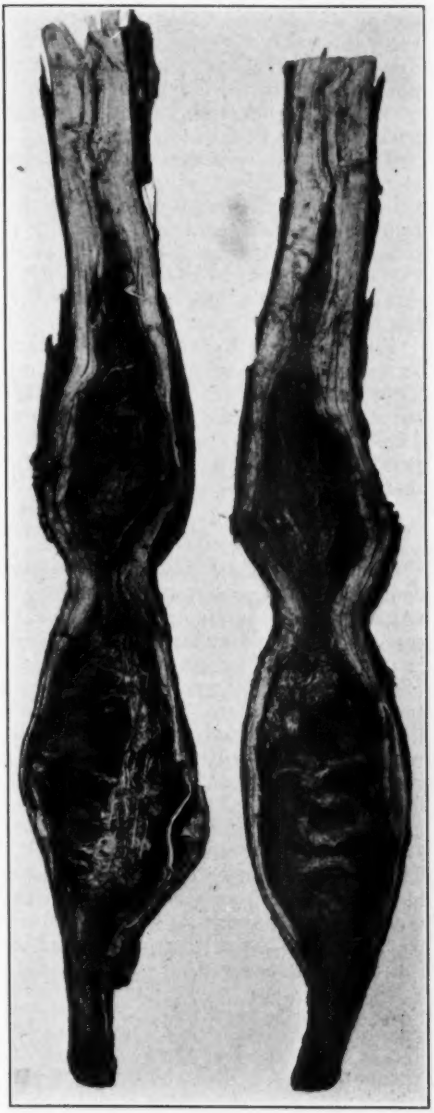

FIGURE 7.—Dried root of *Euphorbia intisy*, showing transverse plates formed by the shrinking of the aquiferous tissue. × ⅓

Although the manner of development of the hydriarhizas seems fairly clear, the actual mechanics involved is not at all understood and apparently it has not been investigated by any worker. Why during wet periods water should move with such disruptive force into these, and only these, portions of the root, and what it is that in the dry season effectively guards against too rapid water loss due to the pull exerted by the completely air-dry soil around the roots and the transpiration demands of the stem above, are questions which offer an interesting field of study for the physiologist.

Though these characteristic water-storage organs are able to withstand a tremendous amount of drought without injury, any rupture that exposes the aquiferous tissue to microorganisms is promptly followed by disintegration. Usually the entire hydriarhiza affected rots away, and frequently the decay extends along the stem, killing the entire plant; occasionally, however, only the aquiferous tissue disappears and a new hydriarhiza forms inside the hollow shell of the old one.

These peculiar hydriarhizas, which may reach a diameter of 15 cm. or even more, constitute an extremely valuable water reservoir. They undoubtedly are the chief factor that enables intisy, a plant that stores practically no water in the stem, to withstand the extremely arid conditions of southern Madagascar, where usually a 6-month period without rain occurs annually and where droughts lasting as many years are not unknown (*9*).

SUMMARY

Euphorbia intisy, a valuable rubber-producing plant, native in southern Madagascar, and first brought to the United States by the writer in 1928, is an upright, woody, almost leafless shrub or tree, reported to attain a maximum height of 7 meters. Its stem structure is characterized by early lignification, delayed phellogen formation, and the presence of very long, ramified latex cells that yield one of the best rubbers known. Certain irregularities of nodal anatomy are noted, one of which may possibly be of specific importance. The root system is unique. In addition to absorbing roots, which display six primary bundles, pericyclic cork, and other common characteristics of the roots of the genus Euphorbia, all of the main roots display alternate series of ordinarily thickened root segments and greatly swollen water-storage segments, here given the name hydriarhizas. These result from an enormous water intake by the cells of the pith-like axial tissue, certain of the primary bundles, the primary and secondary xylem rays, and the xylem parenchyma. Such storage of water enables the plant to withstand the extremely arid conditions of southern Madagascar.

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RÔLE OF CHLORINE IN NUTRITION AND GROWTH OF THE TOBACCO PLANT AND ITS EFFECT ON THE QUALITY OF THE CURED LEAF[1]

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INTRODUCTION

The effect on crop yields of applying to the soil common salt, or sodium chloride, and other chlorides has been a subject of considerable experimentation over many years. Numerous experiments likewise have been made with pot and water cultures to determine whether chlorine is an indispensable nutrient for higher plants and to learn more of its supposed action in stimulating growth. In general, the results have been so distinctly contradictory that no agreement has been reached as to whether chlorine is an essential element. It appears, however, that if it be necessary for normal growth and development the quantity required must be quite small. On the other hand, it is undoubtedly true that under suitable conditions the growth of some plants may be materially stimulated by the presence of proper quantities of chlorine in the soil or other culture medium. It seems that different plants vary considerably in their response to this element. The fairly extensive literature which has been developed on the effects of chlorine on plant growth generally has been reviewed by Tottingham (*34*)[2] and more recently by Lomanitz (*22*), and it is not necessary for present purposes to go further into the subject.

Until quite recently interest in the effects of chlorine on tobacco has centered almost exclusively on its relation to the combustibility of the cured leaf, little or no consideration being given to effects on growth of the plant. The object of this paper is to present data bearing on the effect of chlorine in stimulating growth in tobacco, its rôle in influencing water relations and nutritional processes in the plant, and its relation to color, combustibility, and other characteristics of the cured leaf.

EFFECTS OF CHLORINE ON YIELD OF TOBACCO

Prior to the beginning of the experiments to be considered herein, about the only data tending to establish a significant effect of chlorine on the yield of tobacco were furnished by fertilizer tests conducted over a period of years at the southwestern test farm of the Ohio Agricultural Experiment Station, located at Germantown (*33*). The soil is designated as Miami clay loam. In a 3-year rotation of tobacco,

[1] Received for publication Dec. 11, 1929; issued April, 1930. Cooperative investigations by the Office of Tobacco and Plant Nutrition, Bureau of Plant Industry, U. S. Department of Agriculture, the North Carolina Department of Agriculture and Agricultural Experiment Station, and the Agricultural Experiment Station of the University of Maryland.

[2] Reference is made by number (italic) to "Literature cited," p. 646.

wheat, and clover, with a fertilizer applied to the tobacco crop which furnished 38 pounds of nitrogen, 30 pounds of phosphorus, and 75 pounds of potassium per acre, comparison is made of sulphate, nitrate, and muriate of potash. For the first 12 years (1903–1914) the average yields of tobacco produced with the three forms of potash in the order mentioned were, respectively, 1,213, 1,204, and 1,352 pounds per acre. Thus the muriate showed a gain of about 145 pounds over the sulphate and nitrate. Where lime was applied in addition to the fertilizer, the respective yields with sulphate and muriate were 1,175 and 1,200 pounds. For the succeeding period of 9 years (1915–1923) the average yields obtained with the sulphate, nitrate, and muriate were 1,025, 1,040, and 1,069 pounds, respectively. On the limed plots the sulphate produced 957 pounds and the muriate 947 pounds. Obviously the initial increases obtained with the muriate have not been maintained. Moreover, the initial gain in yield from the muriate is not so pronounced where lime was applied to the soil.

In connection with an extensive series of fertilizer tests with flue-cured tobacco, conducted on the Durham sandy loam and coarse sandy loam soil types at the Tobacco Branch Station of the North Carolina Department of Agriculture located at Oxford, provision was made for field study of the comparative values of different forms of potash, with special reference to the sulphate and the muriate. It subsequently developed that the magnesium supply is an important limiting factor in crop yields on the soils in question, and it became necessary to make provision for this element in the study of the potash salts. Similar tests with potash salts were carried out for a time on Durham sandy loam soil at Reidsville, N. C.

In the first series at Oxford, beginning with 1913, comparison was made with the sulphate and muriate of potash at rates to supply 80 pounds of potash per acre in conjunction with 40 pounds of ammonia derived from dried blood and 64 pounds of phosphoric acid derived from superphosphate. The plots were run in duplicate and were located on three separate fields, each of which was run in a 3-year rotation of tobacco, oats, and corn prior to 1919; subsequently rye harvested for grain was substituted for the corn crop. The oats received 100 pounds of nitrate of soda per acre as fertilizer; the corn was fertilized with 100 pounds each of nitrate of soda and cottonseed meal and 200 pounds of superphosphate; the rye was not fertilized. Beginning with 1920, one-half of each plot received dolomitic limestone broadcast at the rate of 1 ton per acre every third year, prior to transplanting tobacco. The soil type was Durham coarse sandy loam.

On the same soil type a second series of tests was run with the sulphate and muriate salts applied at rates to supply 36 pounds of potash per acre in conjunction with the same supply of ammonia and phosphoric acid as in the first series. Two different sources of muriate and sulphate, namely, domestic and imported, were used. Tobacco was grown each year on the same plots, and the treatments were not duplicated. However, the tests were run in three sections, namely, without addition of limestone, and with 1,000 pounds each of dolomitic and calcitic limestone applied in the drill each year.

These tests also were conducted for a shorter period on Durham sandy loam soil at Reidsville.

The third series at Oxford involves a comparison of sulphate and muriate applied at rates to supply 12, 24, 36, and 80 pounds of potash

per acre. The supply of ammonia and phosphoric acid was the same as in the first series, except that a dicalcic phosphate was substituted for superphosphate. For the first three years the treatments were duplicated, but thereafter one plot of each treatment received every third year one ton of dolomitic limestone. Tobacco was grown on the same plots every year, and the soil was Durham sandy loam. This series also was duplicated on the same soil type at Reidsville, the only difference in treatment being that superphosphate was used in place of dicalcic phosphate.

The detailed results of the above-described tests have been presented in large part elsewhere (*25*), though not with special reference to the effects of chlorine. It will suffice for present purposes to present only the average results of these tests, which have extended over periods ranging from 3 to 14 years. The data are shown in Table 1.

TABLE 1.—*Effect of chlorine on yield and value of tobacco, as indicated by comparative results with equivalent quantities of high-grade sulphate and muriate of potash, with and without application of limestone*

Locality, soil type, series, and treatment No.	Duration of tests	Kind of lime applied	Potash applied per acre	Yield of tobacco per acre with potash supplied as—		Price per 100 pounds of tobacco when potash was supplied as—	
				Sulphate	Muriate	Sulphate	Muriate
Oxford, N. C.; Durham coarse sandy loam:							
Series 1—	*Years*		*Pounds*	*Pounds*	*Pounds*	*Dollars*	*Dollars*
1, 2	14	None	80	754	852	18.50	21.40
3, 4	7	Dolomite	80	972	1,036	20.35	21.68
Series 2:							
1, 2	6	None	36	567	704	24.50	24.91
3, 4	6	Calcite	36	630	644	22.40	24.01
5, 6	6	Dolomite	36	848	929	27.85	29.40
Reidsville, N. C.; Durham sandy loam:							
Series 1—							
1, 2	3	None	36	921	914	15.53	19.34
3, 4	3	Calcite	36	932	1,049	17.95	18.42
5, 6	3	Dolomite	36	937	1,006	18.92	18.72
Oxford; Durham sandy loam:							
Series 3—							
1, 2	10	None	12	491	589	25.65	27.72
3, 4	10	do	24	599	668	26.45	30.56
5, 6	10	do	36	593	698	27.07	30.89
7, 8	10	do	80	643	698	25.79	29.24
Series 4—							
1	7	Dolomite	12	674	687	23.18	25.30
2	7	do	24	695	814	23.80	25.96
3	7	do	36	807	839	26.10	24.74
4	7	do	80	652	796	24.78	26.34
Reidsville; Durham sandy loam:							
Series 2—							
1	5	None	12	715	730	15.82	21.16
2	5	do	24	690	760	18.00	19.78
3	5	do	36	786	832	19.05	22.44
4	5	do	80	768	898	18.66	21.90

The results as a whole leave no doubt of the fact that under the conditions muriate of potash tends to give an appreciable increase in yield as compared with the sulphate. Taking into account the number of years covered by the several tests, the replications, and the variations in the treatments, there are in all 193 comparisons of sulphate and muriate, and the average yields under all conditions are 734 and 807 pounds, respectively. The muriate thus shows an average increase over the sulphate of 73 pounds, or 10 per cent. It is not to be expected, of course, that the muriate will show an advantage

over the sulphate on all soils or under all conditions. The results in Table 1 show a similarity to those reported in Ohio, in that addition of limestone to the soil tends to reduce or eliminate the differences in yield between the two forms of potash. There are instances in the table also in which little or no difference in yield is to be seen, even where no lime was used. However, experiments in other localities, together with numerous observations on the crops of growers, indicate that the response in growth of the tobacco plant resulting from application of chlorides is rather general, especially on light soils having a low water-holding capacity. That the increased yield obtainable with muriate of potash is to be attributed to the effects of the chlorine ion is indicated by the facts that sodium chloride also has been found to give a decided increase in growth on the Oxford soils, and, as is later shown, potash applied in the form of sulphate is readily absorbed from these soils by the plant. Moreover, the stimulating effect of chlorine also is seen when ammonium chloride is used in comparison with other sources of fertilizer nitrogen.

The quantity of chlorine required to obtain maximum yields compatible with high quality of product is naturally a matter of practical importance. The effect of a given quantity varies according to the conditions, so that the quantity required for the best results can only be roughly approximated. The data supplied by series 1 and 2 of the tests at Oxford and series 1 of those at Reidsville merely show that at certain given rates of application muriate of potash in most instances gives a larger yield than an equivalent quantity of the sulphate. The results with muriate applied at different rates in series 3 and 4 at Oxford and series 2 at Reidsville, however, indicate on the whole that under the conditions of the tests maximum yields may be attained with the rates supplying 24 to 36 pounds of potash per acre. In the grades of muriate used the content of chlorine ordinarily is only slightly less than the content of potash, so that apparently maximum results are to be obtained with approximately 20 to 30 pounds of chlorine per acre. The parallel results with the sulphate do not indicate that potash was a serious limiting factor at the rates in question. It may be tentatively concluded, therefore, that on light soils of the type here involved the stimulating effects of chlorine on growth may be largely attained by applying about 25 pounds per acre of this element.

RELATIVE AVAILABILITY OF POTASH IN MURIATE AND SULPHATE AND COMPARATIVE READINESS WITH WHICH THE CHLORIDE, SULPHATE, AND PHOSPHATE IONS ARE ABSORBED BY THE PLANT

The increased yields of tobacco obtained with muriate of potash in comparison with the sulphate naturally raise the question whether the potash applied to the soil in the latter form is as readily absorbed by the plant as that applied as muriate. To answer this question, the potash content of the tobacco grown in certain years in some of the previously described tests in series 1 and 3 at Oxford was determined by analysis. Similar data were also obtained on tobacco that received potash in the form of sulphate in series 1 at two rates not previously mentioned, namely, 40 and 160 pounds per acre. The analyses were made to include the effects of the sulphate and muriate on the absorption by the plant of calcium, magnesium, sulphur, and chlorine. The results are shown in Table 2.

TABLE 2.—*Comparative effects of high-grade sulphate and muriate of potash applied as fertilizer at different rates on the content of potash, lime, magnesia, sulphur, and chlorine in tobacco grown on sandy loam soil at Oxford, N. C.*

Series and treatment No.	Potash applied		Part of plant analyzed	Mineral constituents of tobacco					
	Form	Quantity per acre		K_2O	CaO	MgO	Total sulphur (as SO_3)	Sulphate sulphur (SO_3)	Cl
Series 1:		*Pounds*		*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cen*
1	Sulphate	80	Leaf	3.59	3.29	0.49	1.65	1.03	0.39
2	Muriate	80	do	3.45	3.39	.58	.88	.35	3.58
5	Sulphate	40	do	3.05			1.55	1.08	.36
6	do	160	do	4.96	2.67	.44	2.00	1.38	.80
Series 3:									
3	Muriate	12	do	1.38					
4	Sulphate	12	do	1.26					
5	do	36	do	2.78	4.84	.33	.95	.58	.44
6	Muriate	36	do	2.22	5.14	.45	.63	.28	1.95
7	Sulphate	80	do	3.36			1.13	.65	.83
8	Muriate	80	do	3.11			.55	.15	3.19
5	Sulphate	36	Stalk	1.90	1.18	.23	.48	.28	.45
6	Muriate	36	do	2.00	1.15	.18	.35	.15	1.00
7	Sulphate	80	do	3.32			.95	.63	1.67
8	Muriate	80	do	3.46			.48	.20	3.40

It will be seen at once that there is no substantial difference in the potash content of the tobacco where the two potash salts are used in equivalent quantities. The sulphate tends to give a slightly higher percentage of potash in the leaf, but this is fully offset by the fact that a larger yield of leaf is produced by the muriate. Evidently the differences in yield are not due to difference in availability of the potash supplied by the two salts. It is seen, also, that with both salts increase in quantity of potash added to the soil results in increased content of potash in the tobacco.

Increase in quantity of the two potash salts applied to the soil resulted in progressive decrease in calcium content of the tobacco, but there was no decided difference between the action of the two salts in this respect. The situation is different, however, in the case of magnesium. The Oxford soils are markedly deficient in magnesium, and in all cases the content of this element in the tobacco is low. Increasing quantities of sulphate of potash did not greatly affect the absorption of magnesium, but in each instance the muriate in comparison with the sulphate enabled the plant to absorb a greater supply of magnesium. It was, in fact, the striking difference in effects of sulphate and muriate on visible symptoms of sand drown (magnesium hunger) in tobacco in these tests which first directed attention to the prevalence of magnesium-deficiency symptoms in tobacco grown on light sandy and sandy loam soils. In a study of the effects of different fertilizer treatments on the composition of tobacco in connection with the plot tests at Germantown, Ohio, previously mentioned, Ames and Boltz (*1*) likewise found a definite increase in magnesium content of tobacco when muriate of potash replaced sulphate or nitrate of potash in the fertilizer, although in this case the soil evidently was reasonably well supplied with magnesium. It seems, therefore, that muriate of potash increases the solubility of the magnesium present in the soil, but obviously when the total magnesium supply of the soil is quite low the solvent action of the chloride can hardly be expected to persist over many years. In the case of the Oxford soils it has been found, in fact ,that after a few years'

continued use muriate of potash ceases to be effective in preventing symptoms of magnesium deficiency.

It seems reasonably clear that, on some soils at least, the increased yield obtained with muriate in comparison with other forms of potash is due in part to the solvent action of the muriate on the magnesium supply of the soil, thus enabling the plant to obtain more nearly its minimum magnesium requirements. This would be in accord with the observations of Ames and Boltz (*1*) and more recently those of Anderson and Swanback (*2*) that applying magnesium limestone to the soil increases the magnesium content of tobacco at the expense of calcium and potassium. Thus a possible explanation is found of the fact that the muriate of potash is likely to be less effective in increasing yield when magnesium limestone has been applied to the soil, for both treatments tend to accomplish the same result.

Turning to the absorption of the sulphate and chloride ions by the plant, the data in Table 2 show that in all cases the sulphate of potash increases the content of total sulphur and sulphate sulphur in the tobacco, while the muriate increases the chlorine content. However, the gains in sulphur compounds are relatively small, while the increases in chlorine content are quite large. These results are supported by numerous observations of investigators on other plants. Thus Hoagland and Davis (*19*) found that even in water cultures the chloride ion is much more readily absorbed than the sulphate ion. In the tests with tobacco under consideration it will be observed that when the muriate is used the tobacco contains approximately as much chlorine as potash, whereas the use of the sulphate in equivalent quantities results in a much lower sulphur content. These facts afford additional evidence that the stimulating action of chlorides on plant growth is due, at least in part, to actual absorption of chloride ions.

That the chloride ion is absorbed by tobacco with great ease has been confirmed by a number of additional tests. In one instance tobacco seedlings that had been liberally fertilized with a low-grade muriate of potash were found to contain more than 10 per cent chlorine. In the experiments under discussion the rate of application of phosphoric acid was uniform, but numerous published data show that the capacity of the tobacco plant to absorb the phosphate ion is quite limited and that the phosphoric-acid content of the leaf rarely exceeds 1 per cent. Anderson, Morgan, and Nelson (*3*) report that with fertilizer applications of phosphoric acid ranging from 53 to 250 pounds per acre the range in phosphoric acid in the cured leaf was only 0.61 to 0.65 per cent for the light grade and 0.70 to 0.83 per cent for the dark grade. Ames and Boltz (*1*) likewise found that the use of 480 pounds of superphosphate per acre had practically no effect on the phosphoric-acid content of the leaf.

RELATION OF CHLORINE TO DROUGHT RESISTANCE IN TOBACCO

In the tests with sulphate and muriate of potash at Oxford, N. C., independently of characteristic symptoms of magnesium deficiency, certain clearly defined differences in growth and appearance of the plants fertilized with the two salts could be seen. Where the sulphate was used the leaves of the plants showed a relatively dark green color and to the touch seemed to be comparatively thick. The leaves were relatively small in size. Where the muriate salt was used the leaves

possessed a bright, rather light green color and were thinner and somewhat larger in size. As the plants approached maturity there was at times a tendency toward physiological breakdown in the leaf where the sulphate had been used in the fertilizer. This breakdown appeared in the form of numerous dead spots and blisters, and occasionally also the margins of the leaves withered and died. These symptoms were more pronounced in dry weather, especially when the dry weather followed a period of wet weather. There was little or no evidence of this breakdown when muriate of potash was substituted for the sulphate. The physiological breakdown that has been observed in the leaves of the plants on the sulphate plots appears to be identical with the disease that Fromme and Wingard (*14*) have designated as "drought spot."

A striking instance of the serious proportions which this drought effect may assume and of the definite protective action afforded by chlorine was seen in the tobacco crop produced in the vicinity of Oxford, N. C., in 1925. After the crop had been set in the field and had made considerable growth a severe drought prevailed for some weeks. In many instances the margins of the lower leaves of the plants dried, turned brown, and eventually dropped off. After rain had fallen, subsequent growth of the crop was normal. The soils commonly employed in growing the type of tobacco in question are of a light, sandy character and have a low water-holding capacity, so that in the absence of occasional showers they are likely to become hot and quite dry. In these circumstances the tobacco plant naturally experiences difficulty in maintaining its water supply.

Upon the conditions that prevailed in 1925 striking differences were observed between the experimental plots at Oxford that were fertilized with sulphate and with muriate of potash, respectively. All of the data in Table 2 for series 1 and those for treatments 7 and 8 of series 3 were obtained from material grown under the above-mentioned conditions. The burning of the margins of the leaf, popularly spoken of as "rim fire," was quite severe on all the sulphate treatments in both series except where the potash application was 160 pounds per acre. Except in the lowest rate of application, the muriate treatments prevented any material injury from burning. Potash as such failed to protect the leaves from burning when present in the tissues to the extent of 3.5 per cent of the dry weight and accompanied by a chlorine content of less than 1 per cent. When the chlorine content was increased to 3 per cent or more, evidence of injury was practically absent, and with a chlorine content of about 2 per cent (treatment 6, series 3) there was only slight damage. Again, with a chlorine content of about 0.8 per cent but with an increase in potash content to 5 per cent (treatment 6, series 1) there was effective protection. It appears that both potash and chlorine exercise a protective action against drought, but the latter seems to be decidedly more effective than the former.

The protective action of muriate of potash as compared with the sulphate against injury from drought has been observed in a number of instances in the crops of growers. Several cases were seen in the vicinity of Oxford in 1925. Where the fertilizer did not contain significant quantities of chlorine, the margins of the leaves died off and numerous blisters or spots developed between the larger veins. Similar observations were made in different localities in 1928 where

exceptionally heavy rains were followed by a period of hot sunshine. It must be recognized that injury of the sort in question may subsequently result as a sequel to heavy rainfall or prolonged wet weather as well as directly from prolonged drought.

If it be true that chlorine in the tissues enables the leaf to resist injurious or fatal drying under certain conditions, it should be possible to show that the water content of the leaf is measurably increased under these conditions. Considerable data tending to demonstrate that this is true have been obtained at Upper Marlboro, Md. In 1926 a series of observations was made through the growing season on the water content of the leaves of plants when equivalent quantities of both potash and magnesia were applied in the form of sulphate and chloride, respectively. Similar observations also were made on the leaves of plants fertilized with ammonium sulphate and ammonium chloride, respectively, at rates to supply a fixed quantity of nitrogen. In the first case 64 pounds of phosphoric acid per acre, and in the second case 60 pounds of this nutrient, were included in the fertilizer in the form of dicalcic phosphate. The tests with the potash salts were located on Collington loamy sand, and those with the ammonia salts on Collington fine sandy loam. The first samples of leaf were taken on July 26, 18 days before the plants in the tests with sulphate and muriate of potash were topped. In the other series of tests the plants were not topped till the first blossom opened, which occurred August 26. On each plot the moisture content of the soil to a depth of 6 inches was determined each time the leaf samples were taken. The results of the observations are shown in Table 3.

TABLE 3.—*Effects of chlorine in fertilizer on water content of tobacco leaves during the growing season at Upper Marlboro, Md.*

Plot No.	Nitrogen in fertilizer		Potash in fertilizer		Quantity of chlorine in fertilizer per acre	Water content of leaves						
	Form	Quantity per acre	Form	Quantity per acre		July 26	Aug. 3	Aug. 10	Aug. 26	Sept. 2	Sept. 9	Average
		Lbs.		*Lbs.*	*Lbs.*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
19	Ammonium sulphate.	80	Sulphate	40	0	81.68	85.66	82.20	84.23	83.77	83.49	83.50
20	Ammonium chloride.	80	do	40	202	85.35	88.88	84.38	86.73	85.99	84.40	85.96
10*	Dried blood	40	Muriate	80	130	86.14	89.37	83.04	85.16	81.16	80.19	84.18
11*	do	40	Sulphate	80	0	82.76	85.76	79.49	82.39	77.32	78.78	81.08
						Water content of soil						
19	Ammonium sulphate.	80	do	40	0	7.51	6.78	4.70	10.80		9.27	7.81
20	Ammonium chloride.	80	do	40	202	7.63	6.76	4.92	11.19		9.05	7.91
10	Dried blood	40	Muriate	80	130	3.52	3.55	2.14	8.21	3.72	6.38	4.59
11	do	40	Sulphate	80	0	3.52	3.54	2.04	7.91	4.26	6.11	4.56

* Plot 10 also received 40 pounds MgO per acre in the form of chloride, and plot 11 received a like quantity of MgO in the form of sulphate.

It will be observed that in both cases the addition of 100 to 200 pounds of chlorine per acre consistently maintained a rather marked increase in water content in the leaf throughout the growing season.

These results, which have been confirmed by a number of additional observations, show that chlorine does enable the tobacco leaf to resist desiccation, and this effect might easily account for the increase in yield of the tobacco crop often obtained when the fertilizer contains a considerable percentage of chlorine. There is considerable difference in the water content of the two soil types used in the tests, and there seems to be a relation between this difference in the moisture content of the soil and the water content of the leaf. Also, variation in soil moisture through the season appears to cause considerable variation in water content of the leaf. Finally, there appears to be on the whole a downward trend in water content of the leaves as they grow older. However, the distinctive effect of the chlorine is always in evidence.

EFFECT OF CHLORINE ON CARBOHYDRATE METABOLISM IN THE PLANT

In the preceding paragraphs it has been shown that the presence of chlorine in the soil may enable the plant to absorb more readily the necessary quantity of magnesium to meet nutrition requirements, while the presence of chlorine in the tissues of the leaf enables it to resist injurious desiccation. Thus, the growth of the plant may be stimulated and the yield of leaf may be increased. It now remains to show that a too liberal supply of chlorine in the soil may produce decided injury to the plant, apparently not by direct toxic action but by serious interference with carbohydrate metabolism.

Here, again, it seems that the water relations of the plant are involved. The influence of the water content on carbohydrate metabolism in the plant was first demonstrated in 1914. In that year, in connection with studies on tobacco curing, it was shown in the Office of Tobacco and Plant Nutrition (*16*) that partial wilting of the green leaf has a decided effect in stimulating the enzymatic processes involved in conversion of starch into reducing sugars. In the same year Lundegårdh (*23*) also found that under favorable conditions decrease in water content of plant tissues increase the transformation of starch into sugars, and, conversely, increases in water content causes re-formation of starch. These reactions have since been studied in some detail by Schroeder and his associates (*32*), including Bruns (*7*), and have been shown to have wide application. It was found that in the presence of starch, saccharose is formed as the water content decreases, so that the content of saccharose in the tissues is a function of the water content. This relation holds true in light and in darkness. In darkness the hexose content of the plant tissues also increases with decrease in water content. It seems clear that these transformations in relation to water content are of fundamental importance in the nutrition processes of the plant.

When the quantity of chlorine applied to the soil is in excess of 100 pounds per acre, and in some cases when the quantity used is as low as 40 to 60 pounds per acre, instead of the generally beneficial effects obtained with lighter applications the appearance and development of the leaf are commonly modified to a striking degree. The leaf surface has a sleek, exceptionally smooth or glabrous appearance. To one familiar with this effect of chlorine the appearance of the leaf is quite distinctive and easily recognized. In extreme cases the margins of the leaves curve upward in a characteristic fashion. (Fig. 1.)

Perhaps the most striking characteristic of such leaves is their extraordinary thickness and brittleness. The contrast in thickness between normal leaves and those showing the typical chlorine effect can be seen in Figure 2. The thickened leaf is from a plant that received a liberal application of chlorine in the form of calcium chloride. As seen in cross section, the thickness is several times that of the normal leaf. The leaf cells become gorged with starch, and the tissues are quite brittle. In all the cases studied these evidences of malnutrition have been associated with a high content of chlorine in the tissues, namely, 4 to 7 per cent or more. The malnutrition symptoms have been obtained in pot tests as well as in the field, and with the chlorides of potassium, sodium, and calcium, ammonium chloride, and hydrochloric acid.

When the available supply of chlorine is sufficient to produce the above-described nutritional disturbances to a marked degree, the growth of the plant may be checked. A number of such instances have been observed in the field, and in some cases applications to plant beds of fertilizers containing high percentages of chlorine have resulted in the death of the tobacco seedlings. It appears that the character of the soil and the weather conditions have a material effect on the quantity of chlorine required to produce significant injury to the tobacco crop in the field. On some soils applications of as much as 75 pounds per acre of chlorine have failed to produce notable injury, while, as already indicated, on the other soils material damage has resulted from considerably lower rates of application. Apparently, chlorine injury in the field is most likely to occur on light sandy soils which have only slight buffering properties. That weather conditions also greatly affect the results, even on the same soil, is shown in the following paragraphs.

FIGURE 1.—Tobacco plant in the field, showing marked curling of the leaves as a result of absorption of an excessive quantity of chlorine from the fertilizer used

To obtain information as to the effect of chlorine on the chemical composition of leaf in association with the extreme thickening, partial analyses were made of green leaves collected from plants receiving treatments 10 and 11 at Upper Marlboro, Md., the details of which already have been given. As indicated in Table 3, plot 10 received about 130 pounds of chlorine per acre. In 1923 the leaves of the plants on this plot showed the characteristic effect of a high chlorine content, while in 1924 these effects were not pronounced, apparently because much of the chlorine added in the fertilizer was lost to the plant as a result of leaching. The fresh leaves collected in 1923 and 1924 were quickly weighed and the areas then traced on paper. After removing the midribs, the half leaves remaining were exposed to

chloroform vapors to prevent further changes in composition and then dried at air temperature. The organic acids were determined by the method of Kissling, while the methods of the Association of Official Agricultural Chemists were employed for the other determinations. The results are summarized in Table 4.

TABLE 4.—*Effect of a high chlorine content in the plant tissues on the weight, water content, size, and chemical composition of tobacco leaves grown at Upper Marlboro, Md.*

Year and plot No.	Quantity of chlorine in fertilizer per acre	Total area of 10 leaves	Weight of 10 leaves		Weight of leaf per square foot		Weight of water per square foot of leaf	Water content of leaves	Dry material in leaves
			Fresh	Dry	Fresh	Dry			
	Pounds	*Sq. feet*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Per ct.*	*Per ct.*
1923 {10	130	14.63	524.83	82.56	35.874	5.643	30.231	84.27	15.73
1923 {11	0	14.58	363.58	68.62	24.937	4.706	20.231	81.13	18.87
1924 {10	130	10.00	367.67	65.50	36.767	6.550	30.217	82.19	17.81
1924 {11	0	8.99	323.00	60.70	35.929	6.752	29.177	81.21	18.79

Year and plot No.	Composition of dry matter in leaves							Weight of constituents in 10 square feet dry leaf					
	Chlorine	Starch	Sucrose	Reducing sugars (as invert)	Organic acids	Protein	Pure ash	Starch	Sucrose	Reducing sugars (as invert)	Organic acids	Protein	Pure ash
	P. ct.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*	*Gms.*
1923 {10	4.80	33.51	0.03	3.93	1.58	10.12	------	18.91	0.02	2.22	0.89	5.71	------
1923 {11	.22	24.98	.14	4.31	3.96	11.12	------	11.76	.07	2.03	1.86	5.23	------
1924 {10	1.18	28.74	.00	7.13	3.47	11.50	7.59	18.82	.00	4.67	2.27	7.53	4.97
1924 {11	.10	27.53	.04	6.89	4.99	11.44	7.61	18.59	.03	4.65	3.37	7.72	5.14

The fact that on plot 10, which received more than 100 pounds of chlorine per acre, the leaves of the plants failed to show in 1924 the striking peculiarities in appearance and abnormal thickening observed in 1923 is readily explained by the decided difference in chlorine content of the leaves in the two seasons. In 1924 the leaves contained only a little more than 1 per cent of chlorine, whereas in 1923, with the same quantity of chlorine added to the soil, the content of the leaves was fully four times as high. The effects of the chlorine in the two years were quite different. With slightly more than 1 per cent of this element in the leaf tissues in 1924, the effect was to increase considerably the area of the leaf, while per unit of area the water content, the fresh weight, and the dry weight were only slightly changed. In other words, the increase in total fresh weight and in dry weight of the leaf was due almost entirely to increase in the total area and not to increase in thickness of the leaf. With an increase in chlorine content of the leaves to nearly 5 per cent in 1923 there was no material effect on total leaf area, but the water content and the fresh weight per unit of area were greatly increased and the dry weight also was materially increased. Manifestly, the green leaf was much thickened. Measurements on the cured leaf of the 1923 crop showed an increase of one-third in thickness of leaf from plot 10 over that of plot 11.

With respect to chemical composition, about the only clearly evident effect of the chlorine in 1924 was to reduce the content of organic acids in the leaf, an effect which will be discussed in later paragraphs. In 1923, however, in association with the more decided action of the chlorine in maintaining a high water content, there was a marked accumulation of starch in the tissues of the leaf. Observation has shown that this result is typical, and it seems reasonable to suppose that it is but a special case of the general principle that a high water content in the leaf tissues tends to bring about abnormal accumulation of starch. It appears that carbohydrate metabolism is checked and growth of the plant may be practically stopped. It is evident that amylolytic activity is in some way adversely affected. The cause of the accelerated transformation of starch in wilting leaves has been studied by Bruns (*7*), but this problem has not yet been fully solved.

FIGURE 2.—Cross sections of green tobacco leaves, showing marked thickening of the leaf (left) resulting from heavy applications of chlorine to the soil, as compared with the normal leaf (right). The thickened leaves are highly turgid and extremely brittle

To ascertain whether the interference with amylolytic activity resulting from the presence of chlorine in the leaf is directly dependent on a high water content or is of a more permanent nature, observations were made on the comparative rates of starch transformation during the curing process in tobacco leaves taken from field plots 19 and 20 in one series and plots 10 and 11 in another series at Upper Marlboro, Md. The fertilizer treatments of these plots are shown in Table 3. It is well known that rapid depletion of accumulated starch in the leaf is a characteristic feature of curing under normal conditions, and, of course, the harvested leaves also quickly lose a part of their content of water. Immediately after harvesting one-half of each leaf was cut off along the midrib and dropped into boiling alcohol to arrest metabolic processes. The remaining portions of the leaves with midribs attached were placed in a ventilated dark chamber in which a relative humidity of about 82 per cent and a temperature of 80 to 85° F. were maintained. After 68 hours the midrib was removed from each half leaf and the remaining material was dropped into boiling alcohol. Starch and sugars were determined in each sample according to the methods previously mentioned. The results, calculated on a basis of the original fresh weight of the leaves in each case, are shown in Table 5.

TABLE 5.—*Effect of chlorine applied in the fertilizer on starch digestion in tobacco leaves during a curing period of 68 hours, Upper Marlboro, Md.*

Plot No.	Chlorine applied per acre	Leaf material analyzed	Composition of leaf, calculated on basis of original fresh material		
			Starch	Sucrose (as invert)	Reducing sugars (as invert)
	Pounds		*Per cent*	*Per cent*	*Per cent*
10	130	Original	7.36	0.26	0.44
		Partially cured	.94	.86	3.51
11	0	Original	4.91	.18	.67
		Partially cured	.49	.61	2.21
20	202	Original	2.50	.19	.39
		Partially cured	.31	.17	.66
19	0	Original	1.44	.17	.70
		Partially cured	.15	.13	.41

The addition of chlorine to the soil resulted in a decided increase in starch content in the fresh leaf. Partial loss of water by the leaves after harvest, however, was associated with rapid decrease in starch, the rate of decrease being approximately the same as in the leaves from plants that received no chlorine in the fertilizer. Independently of the chlorine factor, the content of sugar at the end of the curing period decreased progressively with decrease in the original content of starch. These results indicate that the presence of chlorine in the tissues does not appreciably interfere with the hydrolysis of starch or the utilization or transport of the products of hydrolysis which normally take place in association with loss of water in the curing process. In other words, the results are in agreement with what is to be expected if the effect of chlorine in causing abnormal accumulation of starch in the tissues of the plant is but a special case of the general tendency for an abnormally high water content in the tissues to check the dissolution and utilization of starch.

INFLUENCE OF CHLORINE ON QUALITY OF CURED LEAF

That the salt content of sea water impairs the free combustibility of tobacco was known to the early settlers in the tidewater region of Virginia. Clayton (*9*), writing in 1688, states:

> Tobacco that grows on low Lands as far as the Salts, tho' the Plant be never overflowed with salt Water, yet the Ground that feeds the Plant being impregnated with salt Water, that Tobacco smoaks not pleasantly, and will scarcely keep Fire; but do all that a Man can, will oft go out, and gives much trouble in frequent lighting the Pipe * * * *.

The first scientific study of the subject was undertaken by Schloesing in 1860 (*31*), who reached the conclusion that the degree of combustibility of the tobacco leaf is more or less proportional to the content of potash existing in the form of salts of malic and citric acids. Nessler (*26*, *27*) found that chlorine in the leaf has an especially injurious effect on its combustibility, while potash tends to offset this unfavorable effect. Much of the subsequent discussion by numerous investigators relative to the combustibility of tobacco has centered around these two theories of Schloesing and Nessler. In reality the two theories are more closely related than might appear on the surface, and that both are correct when properly applied can no longer be doubted.

Without undertaking a detailed discussion of the subject, it will be sufficient to point out that the importance of one or the other or both

of these theories has been demonstrated or supported by the work of Kosutány (*21*), Fesca and Imai (*12*), Van Bemmelen (*6*), Mayer (*24*), Ogier (*28*), Patterson (*29*), Hissink (*18*), Garner (*15*), Wagner and his associates (*35*), Ames and Boltz (*1*), Frear and his associates (*13*), Haley, Nasset, and Olson (*17*), and Anderson, Nelson, and Swanback (*4*). Preissecker (*30*) has made more readily available Pezzolato's analysis of the problem of combustibility in tobacco, in which, also, the importance of both of these factors is recognized. While not in all cases minimizing the significance of chlorine and organic salts of potash, Johnson (*20*), Barth (*5*), Cserháti (*11*), Cohen (*10*), and Carpenter and Allen (*8*) have suggested that their effects may be masked by other factors which play a part in combustibility. It appears unnecessary to present the extensive data that have been obtained in recent years in the Office of Tobacco and Plant Nutrition further demonstrating the injurious effect of chlorine on the combustibility of tobacco. Some of the data have been summarized by Moss and his associates (*25*), and other portions are reserved for consideration in future publications. In fertilizer practice sound application of the principles here involved requires careful consideration of the type of tobacco and its uses in manufacture, the soil, cultural practices, and other pertinent factors. The requirements as to combustibility are most rigid in the case of cigar leaf but are also of considerable importance in all types used for cigarettes and for pipe smoking.

With a given content of potash in the leaf, the proportion of the total that may exist in the form of organic salts depends primarily on the relative content of potash and of chloride and sulphate radicals, although of course the content of lime and magnesia is of more or less moment. The significance of the chlorine-potash ratio, therefore, may be considered as due largely to its inherent bearing on the content of organic potash in the leaf. It has been shown in the present paper that the chloride ion is much more readily absorbed from the soil by the plant than the sulphate ion. The natural effect of applying the chlorides of potassium, sodium, calcium, or magnesium to the soil is a sharp increase in chlorine content of the plant, and the chlorine-potash ratio may be expected to be relatively high. On the other hand, when soluble sulphates are applied to the soil the sulphate ion is absorbed by the plant with difficulty. Moreover, the absorbed sulphate ions in part undergo transformation, entering into the make-up of certain proteins. It is clear that the potassium or other metal occurring in the plant in the form of chloride is not available for the formation of salts of organic acids except as replaced by other metals. In general, therefore, when muriate of potash is used as a fertilizer the chlorine-potash ratio in the tobacco leaf will be high, both the chloride and potassium ions being readily absorbed, while the content of potash salts of organic acids will be comparatively low. Apparently, the latter result explains in part the unfavorable effect of chlorine on the combustibility of tobacco. When sulphate of potash is used as a fertilizer the potassium ion is more readily absorbed than the sulphate ion, so that the sulphate-potash ratio in the leaf is low and a large proportion of the potash is free to combine with organic acids. To a considerable extent sulphate of potash as a fertilizer functions like the carbonate.

Just as in the case of the green plant in the field, chlorine has a decided effect on the water content of the cured tobacco leaf. It has

been frequently observed that when a fertilizer with a high chlorine content is used in growing the crop the cured tobacco leaf retains sufficient moisture for handling without breakage or damage under conditions where other comparable leaf is quite brittle and crumbles when handled. To obtain definite information on this effect of chlorine in the leaf, a number of samples of leaf tobacco grown with and without the addition of chlorine in the fertilizer were exposed to fixed conditions of temperature and relative humidity, and the water content of the samples was determined. The material used consisted of flue-cured leaf from treatments 1 and 2 of series 1 and treatments 7 and 8 of series 3, shown in Table 2; and Maryland leaf from plots 10, 11, 19, and 20, the treatments of which are indicated in Table 3. The tests with material from treatments 7 and 8 of series 3 in Table 2 covered two different years, and in addition to plots 19 and 20 of Table 3 a third plot, in which the nitrogen was derived from nitrate of soda, was included in selecting material. All samples were cured normally except those from plots 10 and 11 of Table 3. In this case the fresh leaves were chloroformed before being dried. For the tests a fixed temperature of 77° F. was used. Small samples of the pulverized leaf were placed for 48 hours in desiccators containing saturated solutions of ammonium nitrate, sodium nitrate, ammonium sulphate, and potassium chloride, respectively. The corresponding relative humidities to which the samples were thus exposed were approximately 62.5, 75.1, 81.8, and 85.8 per cent. To ascertain the moisture content of the samples after the exposures they were first weighed, then dried to constant weight over concentrated sulphuric acid, and again weighed. Finally, the samples were heated in the oven at 101° C. for four hours and weighed. Tobacco contains certain volatile constituents other than water, and for this reason the two different methods of estimating the water content were employed. The results are shown in Table 6.

TABLE 6.—*Effect of fertilizers containing a high percentage of chlorine on the water content of tobacco grown at Oxford, N. C., and Upper Marlboro, Md., when exposed at 77° F. to relative humidities corresponding to the vapor pressures of saturated solutions of certain salts*

Locality and treatment or plot No.	Quantity of chlorine in the fertilizer per acre	Chlorine content of tobacco	Water content of tobacco									
			In air-dried condition		Over NH_4NO_3 solution, relative humidity 62.5		Over $NaNO_3$ solution, relative humidity 75.1		Over $(NH_4)_2SO_4$ solution, relative humidity 81.8		Over KCl solution, relative humidity 85.8	
			By drying over H_2SO_4	By drying in oven	By drying over H_2SO_4	By drying in oven	By drying over H_2SO_4	By drying in oven	By drying over H_2SO_4	By drying in oven	By drying over H_2SO_4	By drying in oven
Oxford, N. C.:	*Lbs.*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Series 1—												
Treatment 1	0	0.39	3.61	6.02	9.56	-------	14.26	16.55	17.78	-------	19.87	22.37
Treatment 2	75	3.58	4.03	7.08	10.78	-------	16.53	19.19	20.78	-------	23.59	26.90
Series 2—												
1918 (1	0	.52	3.35	5.43	7.89	-------	11.98	13.91	14.70	-------	17.12	19.40
1918 (2	75	3.18	3.58	5.78	8.44	-------	13.06	15.25	17.25	-------	19.36	21.60
1925 (1	0	.83	4.43	5.93	8.84	10.45	14.35	16.03	18.64	19.97	23.15	24.56
1925 (2	75	3.19	4.64	6.79	10.35	12.62	17.26	19.50	22.89	24.81	28.62	30.81
Upper Marlboro, Md.:												
Plot 10	130	4.80	4.99	6.92	9.75	11.93	14.62	16.72	16.53	18.29	19.81	21.45
Plot 11	0	.22	4.91	6.87	9.52	11.32	13.95	15.76	15.77	18.06	18.73	20.71
Plot 18	0	.24	5.04	7.80	8.28	11.05	13.85	16.50	16.54	19.26	19.86	22.77
Plot 19	0	.23	4.98	7.49	7.56	10.76	12.74	15.54	15.28	17.09	18.27	20.50
Plot 20	202	5.34	6.07	9.23	10.92	14.12	17.95	20.43	21.47	24.58	25.94	29.27

In all cases the increased content of chlorine has an appreciable effect on the moisture content of the tobacco, although at relative humidities below 60 per cent this effect is slight. As the relative humidity increases, the effect of the chlorine increases and becomes rather pronounced with a humidity of 75 per cent or higher. Where the green leaf was chloroformed so as to prevent normal curing the effect of the chlorine was much reduced even when the relative humidity was high. Moreover, the data for treatments 1 and 2 of series 2 at Oxford, N. C. (Table 6), suggest that with uniform fertilizer treatment on the same soil there may be a distinct seasonal effect on the hygroscopic properties of the tobacco, the product of 1925 having a greater affinity for water than that of 1918.

The question as to the practical advantage or disadvantage of the increased water content of tobacco resulting from an increased content of chlorine is a matter of some complexity. Much depends on the conditions to which the tobacco is exposed, the use to be made of it in manufacture, and the stage that it has reached in handling and manufacturing operations. As the crop leaves the farm it will ordinarily appear on the market with an increased water content. Under relatively dry conditions the increased water content may tend to give the leaf a better appearance, to increase its elasticity and richness or "oiliness," and to prevent damage from breakage. In damp weather the tendency will be to bring the leaf into very high order and increase the danger of damage from excess moisture. These relationships also will apply more or less to manufactured tobacco products as well as to the leaf. In the case of cigarette, pipe-smoking, and chewing leaf there will be, of course, an increased shrinkage in weight in the redrying process. Finally, it has been found that the moisture content of tobacco materially affects its combustibility, and increased content of moisture is probably one of the factors in the adverse effect of chlorine in the leaf on its combustibility.

The chlorine content often affects the color of the tobacco leaf. As previously noted, a moderate chlorine content tends to produce a lighter shade of green in the leaf in the field, and in the case of flue-cured tobacco the cured leaf often shows a lighter shade of yellow. In the latter case, however, the luster may be somewhat dimmed, the leaf surface showing a more or less faded appearance. These effects are not quite so apparent in most other types of tobacco. With a higher content of chlorine the influence on color of the cured leaf may be decidedly injurious, the leaf surface being more or less splotched with areas of green and yellow and the appearance as a whole distinctly abnormal. The leaf also is often lacking in toughness and elasticity and readily breaks when stretched.

It remains to touch briefly upon the effect of chlorine on the actual market price of leaf tobacco under existing marketing conditions. In the sale of most leaf tobacco, relative values of different grades or qualities of a given type are arrived at by the expert buyer simply by inspecting and feeling the product and on occasion noting its odor. In a broad way, relative values usually, though not always, may be determined with a fair degree of accuracy by this procedure. This is true because the buyer through long experience has learned to associate the characteristics measured by the sense of touch and the color and general appearance of the leaf with certain elements

of quality required in the manufactured product. However, this method of arriving at relative values can not be relied upon to reflect the true worth of the product for manufacturing purposes where the effects of special treatments, such as differences in fertilizer used, are involved, since the buyer is not always in a position to recognize or interpret the effects in question. This applies with special force to differences in combustibility and in aroma and, in some instances, in keeping qualities of the leaf. For example, one of the most valuable characteristics of Maryland tobacco as a whole is its good burning qualities. In tests at Upper Marlboro ammonium chloride has been used at varying rates in the fertilizer in comparison with other forms of nitrogen. In these tests expert buyers have at times assigned the highest valuation to the product grown with ammonium chloride, although the leaf when tested has been found to have such extremely poor burning qualities that it seems probable that if such a fertilizer were used generally in growing the crop the market for this type of leaf would be seriously affected. Moreover, the appearance of the leaf is more or less abnormal, and for this reason the buyers at times have reversed their valuations, assigning a relatively low valuation to this product.

Valuations furnished by experienced buyers are given in Table 1 for flue-cured tobacco grown at Oxford and Reidsville, N. C., over a period of years, with equivalent quantities of sulphate and muriate of potash, with and without the addition of dolomitic or calcitic limestone. The soils in question are deficient in magnesia, and the action of chlorides on the magnesia of the soil already has been discussed. Except where dolomitic limestone was used, the tobacco suffered more or less severely from magnesium deficiency on the plots treated with the sulphate of potash. Injury from this cause was decidedly less marked on the muriate plots in the earlier years of the tests. As an average of 11 comparisons where no limestone was used, the difference in price per 100 pounds of the tobacco was about $3.12, or 12 per cent, in favor of the muriate. That this difference was due in part to magnesium deficiency rather than to the specific effect of chlorine is indicated by the fact that where dolomite was used seven comparisons gave an average increase in value from the muriate of only $1.02, or somewhat less than 5 per cent. With use of calcite two comparisons gave but little advantage of the muriate over the sulphate, probably because of the depressing effect of the calcite on the availability of the magnesia in the soil.

As to the quantity of chlorine in the fertilizer required for obtaining leaf of the best quality as judged by appearance, the data in Table 1 are rather limited and afford only a tentative conclusion. Where dolomitic limestone was not used, maximum results as to quality were obtained with quantities of muriate supplying 20 to 30 pounds of chlorine per acre. Where the limestone was applied there seems to have been little or no significant gain in quality from the use of muriate as compared with sulphate. As has been stated, a high chlorine content in the leaf has an injurious effect on its market value as judged by appearance. This effect is illustrated by the valuations made by buyers on the crops of 1926 and 1927 on plots 10 and 11 (run in duplicate) at Upper Marlboro, Md., the treatments of which are indicated in Table 3. The results are summarized in Table 7. In this instance the buyers' valuations for the leaf grown with the chlorides of potas-

sium and magnesium were only about one-half those for the product grown with the sulphates of potassium and magnesium. The reduced valuations were due primarily to the undesirable colors produced by the chlorine.

TABLE 7.—*Effect of a high chlorine content in the fertilizer on the sale value of tobacco grown at Upper Marlboro, Md., as arrived at by buyers after inspection*

Plot No.	Quantity of chlorine in fertilizer per acre	Value of leaf per 100 pounds		
		1926	1927	Average
	Pounds	*Dollars*	*Dollars*	*Dollars*
10	130	15.75	14.75	15.25
11	0	27.50	31.00	29.25

SUMMARY AND CONCLUSIONS

Although it has long been known that under some conditions common table salt and other chlorides will give measurable increases in crop yields, it has not yet been definitely determined whether chlorine is one of the essential plant nutrients. In the present paper field data are presented which show that on light sandy and sandy loam soils a moderate supply of chlorine in the fertilizer commonly stimulates the growth of the tobacco plant, the average increase in yield obtained experimentally being about 10 per cent. These data are based primarily on tests with sulphate and muriate of potash at rates to supply equal quantities of potash. Where dolomitic limestone was applied to the soil, the gain in yield of tobacco from the chlorine was reduced. Under the conditions of the tests 20 to 30 pounds of chlorine per acre were sufficient to give approximately the maximum stimulating effects of this element. Analyses of the tobacco show that in these tests the potash applied to the soil in the form of sulphate was quite as readily absorbed by the plant as that applied in the form of muriate. However, while the chlorine ion also was readily absorbed by the plant, only a comparatively small proportion of the sulphate ions were taken up. In effect the chloride of potassium may be regarded as entering the plant as such, while the potassium furnished by the sulphate enters largely in some other form. The data indicate that the ease with which the chlorine ion enters the plant is in sharp contrast with the behavior of sulphate and phosphate ions.

It has been found that on the soils employed in these experiments muriate of potash used as a fertilizer results in an increased content of magnesia in the plant. Since these soils are markedly deficient in magnesia, it is probable that the stimulating action of the chlorine in muriate of potash on the growth of tobacco is partly due to its effect in temporarily increasing the availability of the soil's reserve supply of magnesia. It has been observed, in fact, that the immediate effect of the muriate is to reduce decidedly the symptoms of magnesia deficiency in the plant, although in later years this effect may be reversed, as is to be expected where the total supply of magnesia in the soil is low.

The chlorine ion has a marked effect in increasing the water content of the leaf of the tobacco plant through the growing season, thus enabling the leaf to resist injurious desiccation. This is of practical

importance in protecting the leaf against the type of injury known as "drought spot." On light soils of low water-holding capacity this protective action against drought injury may result in adding materially to the commercial value of the crop. The effect of a moderate content of chlorine in the tissues in inducing development of a leaf having a greater spread, a lighter green color, and a smoother surface perhaps may be attributed to the drought-resistant properties imparted by the chlorine. It seems likely that this effect of chlorine in increasing turgidity in the tissues explains, in part at least, the stimulating effect of this element on plant growth which has often been observed in various plants.

While a moderate supply of chlorine in the tissues may be of distinct benefit to the plant in enabling it to resist desiccation and thereby stimulating growth, an excess of this element may seriously interfere with normal carbohydrate metabolism. This effect may be severe enough to check growth, and in the seed bed has been observed to result in the death of young seedlings. Normal amylolytic activity in the leaf is disturbed, with the result that there is marked accumulation of starch and the leaf is greatly thickened. It has been found, moreover, that the water content is abnormally high. The leaf assumes a characteristic appearance, which, as a rule, is easily recognized. This physiological effect of chlorine is possibly a special case of the general principle that the relative concentrations of starch and sugars in the tissues is a function of the water content. Partial loss of water, as in curing, results in rapid depletion of the accumulated starch in the leaf.

The adverse nutritional effects and retarded growth may result from considerably less than 100 pounds of chlorine per acre applied in the fertilizer, and in some cases considerable injury has resulted from only 40 to 60 pounds per acre, although the intensity of the effects from a given quantity of chlorine will necessarily vary with the conditions. Injury from chlorine is most likely to develop or to be intensified on sandy soils having limited buffering properties. On such soils a high content of chlorine in the fertilizer also may cause difficulty in obtaining a good stand of plants. The amount and distribution of rainfall affects both the quantity of chlorine absorbed by the plant and its effect on growth.

Broadly speaking, from the standpoint of quality the chlorine content of the cured leaf may affect its properties either favorably or adversely, depending on the quantity of the chlorine present, the use to be made of the tobacco, and the stage in handling and manufacturing operations which it has reached. The presence of chlorine increases the moisture content of the cured leaf, which may affect more or less its elasticity, its combustibility, and its keeping qualities. Chlorine tends to injure the combustibility of the leaf. This effect is often of great importance but may be more or less obscured by other factors affecting combustibility. A free burn is especially necessary in cigar leaf but is also of considerable moment in the case of cigarette and pipe-smoking types. The adverse effect of chlorine on combustibility is partly due to the fact that its presence reduces the quantity of potash which may exist in the leaf in combination with organic acids. Excess chlorine produces muddy colors in the cured leaf, often with considerable splotching in which green, yellow, and

brown are intermingled. A high chlorine content in the fertilizer tends to injure the toughness and elasticity of the leaf.

As judged by expert buyers on the basis of appearance, leaf tobacco grown with a limited supply of chlorine has shown a moderate increase in market value. Under the conditions of the tests, maximum effects in this direction have been obtained with 20 to 30 pounds of chlorine per acre. It is possible that under some conditions somewhat larger quantities would be required for best results. With an excess of chlorine in the fertilizer, the market value of the leaf has been materially lowered.

To determine the exact quantity of chlorine required in the fertilizer for maximum results as to both yield and quality of tobacco obviously is a complex problem, and a more extensive study of the subject is needed.

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THE TOXIC CONSTITUENT OF RAYLESS GOLDENROD[1]

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INTRODUCTION

Rayless goldenrod or jimmy weed *Aplopappus heterophyllus* (A. Gray) Blake, has for more than 20 years been suspected of causing the sickness known as alkali disease that occurs in western Texas, in the Pecos Valley in New Mexico, and in certain parts of Arizona. This disease was early recognized as clinically identical with the milk sickness of human beings and the trembles of animals, diseases that occur in the Central States and are known to be the result of richweed or white-snakeroot poisoning (*5*).[2] Field experiments by Marsh, Roe, and Clawson demonstrated that feeding the New Mexico plant produces a syndrome identical with results from the ingestion of richweed, and established rayless goldenrod as the etiological factor in alkali disease (*7*, *8*).

A chemical and pharmacological study of rayless goldenrod was undertaken by the writer, and at the same time richweed was subjected to study. The results of the investigation of the latter plant have been published (*2*, *3*, *4*). The poisonous substance responsible for milk sickness and trembles was shown to be an alcoholic compound which was named tremetol. Lack of material prevented a continuation of the study of rayless goldenrod until recently, when a sufficient quantity was obtained to permit the isolation and identification of the active constituent.

METHODS AND MATERIAL

The methods used in this study were the same as those employed in the investigation of richweed previously reported. The plant material was obtained from New Mexico, and except for one lot which was put into milk cans and preserved in denatured alcohol as soon as collected, the plants were air-dried. The extracts from the plants were fractionated and the various fractions were fed to guinea pigs and sheep. Guinea pigs were abandoned as test animals for reasons already given (*2*) and reliance was placed only on the results obtained from experiments with sheep.

The doses given to sheep were calculated to represent as nearly as possible 16 pounds of dried plant per hundredweight of sheep, given in four doses, one each day, but this standard was departed from at times. If the extract was soluble it was given in water solution; otherwise it was emulsified in water with the aid of alkaline sirup of acacia. All doses were administered by drenching the sheep.

GENERAL PROCEDURE IN FRACTIONATING THE PLANT EXTRACT

In the course of this investigation 11 different lots of *Aplopappus heterophyllus* were extracted and the extracts fractionated by chemical

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[2] Reference is made by number (italic) to "Literature cited," p. 658.

means in an effort to determine the active principle. In some cases the methods were altered to provide certain special information, but in general the procedure described below was used. Figure 1 indicates the general methods employed.

The ground and dried plant was moistened with alcohol, packed in a tin-lined copper percolator, flooded with alcohol, allowed to macerate for two days, and then extracted by intermittent percolation with alcohol as long as it yielded soluble matter to that solvent. The yield of extract averaged 10 per cent of the material used. The percolate was collected and distilled, the distillate being returned to

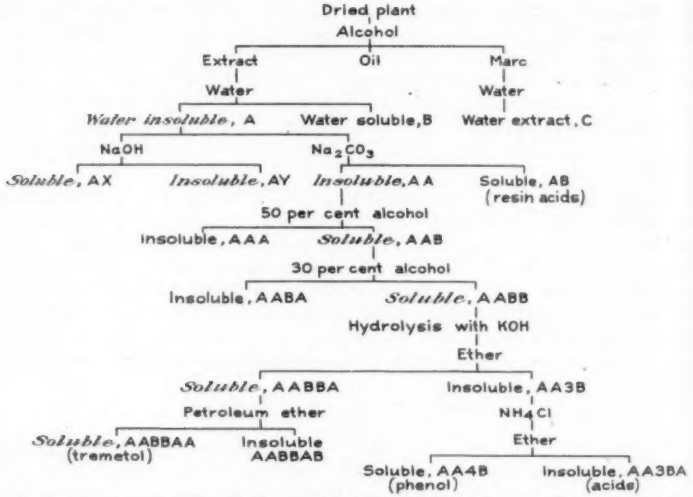

FIGURE 1.—Diagram illustrating the method of fractionating the plant extracts. The active fractions are italicized

the percolator and the residue being frequently poured from the still into a glass receptacle and stored till the end of the process. When the plant had been thoroughly extracted with alcohol the marc was, for one experiment, extracted by water and the water solution was concentrated and used in animal experimentation.

The alcoholic extract was now freed from water-soluble matters by boiling with water in the still, repeating the operation as long as water-soluble substances were removed. This treatment also resulted in the driving off of volatile oil which was condensed and collected. The best yield of oil was from a specimen of plant about a year old and amounted to 0.16 per cent of the weight of the dried plant. The water solutions were collected and concentrated and these constituted fraction B.

The fat and resin portion, insoluble in water, constituted fraction A. In some cases it was further extracted by aqueous sodium carbonate solution which removed resin acids (fraction AB), and in one case aqueous sodium hydroxide was used.

The latter, however, was discarded because it emulsified the trametol and both resulting fractions were toxic. The fat and resin portion (fraction AA) freed from resin acids was then thoroughly ex-

tracted with boiling 50 per cent alcohol in successive portions, dividing the material into two new fractions, an insoluble portion consisting of fats, waxes, resins, and coloring matters (AAA) and a soluble toxic fraction (AAB).

The dissolved matters were recovered from the latter by distilling off the alcohol when they separated from the watery solution in the form of a thick, brownish, balsamic mass (AAB). This was further fractionated by extraction with boiling 30 per cent alcohol which yielded two fractions, an insoluble and nontoxic portion (AABA) and a soluble toxic fraction (AABB).

The latter was hydrolized by boiling with 5 per cent alcoholic potash, the solvent was evaporated off, and the residue was dissolved in water and extracted with several portions of ether. When all had been extracted the ether was distilled off, leaving a brownish oil (fraction AABBA). The KOH solution was then treated with an excess of ammonium chloride and again extracted with ether, which removed phenolic substances (AA4B). The resulting alkaline solution was acidified to recover the acids; the precipitate was filtered off (AA3BA) and the filtrate was extracted by ether to recover soluble acids. The residue from this last ether extraction was added to the acid fraction.

Fraction AABBA contained the tremetol. This was purified by solution in a small quantity of ether and precipitation by four volumes of petroleum ether which threw out much of the coloring matter and contaminating resinous impurities. The ethereal solution was washed with 2 per cent sodium hydroxide solution and then with water, and the solvent was removed. The resulting light-colored oil was redissolved in ether, treated with petroleum ether, dried over fused calcium chloride, and the solvent was removed. This treatment was repeated until the product gave a constant molecular weight, when it was judged pure.

EXPERIMENTS TO DETERMINE THE TOXIC CONSTITUENT

FRACTION C, WATER SOLUBLE

After one lot of plants had been thoroughly extracted by alcohol the marc was extracted by water in order to determine whether the active principle might be insoluble in alcohol and soluble in water. The concentrated water extract was fed to sheep 574 (Table 1) in six doses, each equivalent to 5 pounds of dried plant per hundredweight of animal. There was no apparent effect from this feeding. A similar fraction from another lot was fed to sheep 675 in four doses equal to 4 pounds each per hundredweight without effect.

FRACTION B, ALCOHOL AND WATER SOLUBLE

The water-soluble material obtained by treatment of the alcohol extract as above described reduced alkaline copper solutions, formed a persistent foam, gave Mayer's test for alkaloids, and contained a glucosidal substance that might be precipitated from the solution by making it strongly acid. Four sheep were fed on the whole fraction from different lots. In 1920 sheep 600 and 608 were each given two 5-pound doses without effect. In 1922 sheep 690 was given eight 4-pound doses with the result that it developed some difficulty in breathing and a catarrhal condition that appeared to be due to a traumatic pneumonia. From this the animal recovered. Nothing like trembles was observed.

TABLE 1.—*Summary of experimental results of feeding various fractions of the extract of rayless goldenrod to sheep*

Dates fed	Animal		Fraction fed	Number of doses	Plant equivalent (lbs. per cwt.)		Character or constituents of fraction fed	Tremetol	Symptoms of animal	Termination
	No.	Weight			Per dose	Total				
1920		*Pounds*								
July 1–2	600	81	B	2	5	10	Water soluble	None	None	Survival.
July 7–8	608	79	B	2	5	10	do	do	do	Do.
July 10–14	608	72	AB	4	6.15	24.6	Resin acids	do	do	Do.
Aug. 13–24	574	89	C	6	5	30	Alcohol insoluble	do	do	Do.
1922										
July 23–31	690	85.25	B	8	4	32	Water soluble	do	Sick (pneumonia)	Recovery.
July 27–Aug. 1	698	110	AY	5	4	20	do	Present	Trembles	Death.
Aug. 9–12	676	95.5	AX	4	4	16	do	do	do	Do.
Aug. 5–8	675	108.5	C	4	4	16	Alcohol insoluble	None	None	Survival.
1923										
Oct. 24–27	782	79	AB	4	4	16	Resin acids	do	do	Do.
Nov. 5–8	782	79	AB	4	4	16	do	do	do	Do.
Nov. 14	782	79	AA4B	1	8	8	Phenols	do	do	Do.
Nov. 15–20	782	79	AA4B	5	4	20	do	do	do	Do.
Dec. 11–14	782	79	AB	4	8	32	Resin acids	do	do	Do.
1924										
May 12–16	782	95	AB	4	4	16	do	do	do	Do.
May 17	782	95	AB	1	8	8	do	do	do	Do.
Sept. 15–18	782	99.5	AA4B	4	4	16	Phenols	do	do	Do.
Sept. 22–25	782	99.5		4	4	16	Benzene soluble	Present	Sick	Recovery.
Sept. 26–27	782	99.5		2	8	16	do	do	do	Do.
Oct. 1–6	782	99.5		8	4	32	Benzene insoluble	None	None	Survival.
Oct. 8–29	782	99.5	AA4B	17	4	68	Phenols	do	do	Do.
1925										
May 29–June 3	4	60	AAA	4	4	16	50 per cent alcohol insoluble	do	do	Do.
June 8–11	4	60	AAB	4	6.67	26.67	50 per cent alcohol soluble	Present	Trembles	Recovery.
July 6–11	5	75	AA3BA	6	4	*23	Acids	None	None	Survival.
July 15–18	5	75	AA4B	4	8	32	Phenols	do	do	Do.
July 23–25	5	75	AB	4	6.25	25	Resin acids	do	do	Do.
Aug. 5–8	5	75		2	15.32	30.64	Vacuum fraction 1	do	do	Do.
Oct. 12–15	5	93		4	11.67	46.67	Vacuum fraction 2	do	do	Do.
Nov. 3–7	5	93		5	16.5	82.5	Vacuum residue	do	do	Do.
Nov. 17–20	5	93	AABBAA	4	4.3	17.2	Ligroin soluble	Present	Sick	Recovery.
Dec. 8–19	5	93	AABBAB	11	7.5	82.5	Ligroin insoluble	None	None	Survival.

1926										
June 28–July 1	6	78.5	AABA	4	18.47	73.88	30 per cent alcohol insoluble	do	do	Do.
1927										
Mar. 18–22	8	57	B	4	4	16	Water soluble	do	do	Do.
Do	7	94	AB	4	4	16	Resin acids	do	do	Do.
July 8–16	9	66	AABA	7	5.5	38.5	30 per cent alcohol insoluble	do	do	Do.
July 25–29	8	72	AABBAA	5	4	20	Ligroin soluble	do	do	Do.
Aug. 1–3	8	72	AABBAA	3	8	24	do	do	do	Do.
Aug. 4	8	72	AABBAA	1	16	16	do	do	do	Do.
July 27–Aug. 5	10	60	AABA	4	5	20	30 per cent alcohol insoluble	do	do	Do.

[a] 1 dose of 3 pounds' equivalent.

The same fraction was fed to sheep 8 in 1927, four doses of 4 pounds being administered without effect. Since these feedings demonstrated that the water solution with its contained glucoside could not be regarded as the causative factor in rayless-goldenrod poisoning, nothing further was done with it.

FRACTION AA, FATS AND RESINS

The failure of the water extracts to produce trembles indicated that the active principle is contained in the fraction insoluble in water. This fraction was divided into two portions by the use of aqueous sodium carbonate solution which dissolved the acid constituents of the mass (fraction AB). The matter insoluble in sodium carbonate was a dark-green, unctuous mass of pleasant, balsamic odor. In one experiment sodium hydroxide was used instead of sodium carbonate to effect the separation, and the two fractions (AX, the soluble matters, and AY, the insoluble) were tested. Fraction AX was given to sheep 676 in four doses, each equivalent to 4 pounds per hundredweight of animal. The animal developed a typical case of trembles, excreting acetone by lungs and kidneys, and died five days after the last dose was administered. Fraction AY was given to sheep 698 in five doses, each equivalent to 4 pounds per hundredweight. On the fifth day the animal began to exhibit symptoms of disease and soon presented a typical case of trembles. Acetone was present in the exhaled air and the urine. The sheep died on the second day after the last dose. These two cases indicated that the active principle was present in the water-insoluble fraction but yielded no information as to whether the principle was acid in nature. It was later learned that the active substance had been emulsified by the alkaline solution and that it could be removed from the mixture by means of ether. Four doses of the fraction after extraction with ether, each equivalent to 4 pounds of dried plant per hundredweight of animal, were given to sheep 782 and did not affect the animal.

FRACTION AB, THE RESIN ACIDS

The resin acids were recovered by acidifying the sodium carbonate solution and filtering off the curdy precipitate that appeared. They constituted a brownish mass of soft, resinous consistence from which nothing of a crystalline nature could be obtained. Resin-acid fractions from several lots were fed at different times. In 1920 four doses, each equivalent to 6.15 pounds of dried plant per hundredweight of animal, were administered to sheep 608 without affecting it. In 1923 sheep 782 received four doses, each equivalent to 4 pounds of dried plant per hundredweight on two occasions, then four doses of 8 pounds' equivalent, and in 1924 the same sheep received four doses of 4 pounds' and one of 8 pounds' equivalent and was not affected. In 1927 sheep 7 received four doses of 4 pounds' equivalent without effect.

Later procedure omitted the fractionation by means of sodium carbonate, and the resin acids were fed in mixture with other constituents. In no case, however, was any effect noted that could be attributed to toxicity on the part of the resin acids.

FRACTIONATION WITH 50 PER CENT ALCOHOL

The resinous mass insoluble in sodium carbonate solution and, in later work, the water-insoluble portion of the original alcohol extract were further fractionated by boiling with 50 per cent alcohol, which dissolved approximately one-third of the whole. The insoluble matter was collected as fraction AAA and the dissolved extract was recovered by removing the alcohol from the solution (fraction AAB). Both portions were tested.

The insoluble fraction AAA was given to sheep 4 in four doses, each equivalent to 4 pounds of dried plant per hundredweight of animal, and produced no effect. The other portion (AAB) was administered to sheep 4 in three doses of 6.67 pounds' equivalent when the sheep became sick. A fourth dose of the same quantity was then administered, and the sheep developed a typical case of trembles. She was given a dose of 2 ounces of Epsom salts in 1 pint of water and fed plenty of oats. She gradually recovered and one week later was discharged. Excretion of acetone was not observed in this case.

FRACTIONATION WITH 30 PER CENT ALCOHOL (AAB)

The active fraction was then thoroughly extracted by boiling 30 per cent alcohol, filtering off the hot solvent. On cooling, a brownish viscous mass was deposited which constituted fraction AABB. The undissolved matter from the original fraction was labeled fraction AABA. The latter was administered to sheep 9 in seven doses, each equivalent to 5.5 pounds of dried plant per hundredweight of sheep, and did not affect the animal. It was also administered to sheep 10 in four doses of 5 pounds' equivalent without effect. Sheep 6 received four doses of this fraction, each equivalent to 18.47 pounds of dried plant per hundredweight, without effect.

FRACTIONATION OF THE ACTIVE MATERIAL (AABB)

The quantity of AABB obtained was too small to permit extensive pharmacological study, so the attempt was made to divide it into constituents and to test these separately. The fraction was dissolved in 5 per cent alcoholic potash and boiled on a water bath under reflex until it was hydrolyzed. The alcohol was then distilled off and the residue was dissolved in water and shaken out with successive portions of ether. This extract (AABBA) contained the tremetol and was purified in the usual way. The solution of ether-insoluble matters was treated with an excess of ammonium chloride to liberate the phenol present and again shaken out with ether. The residual aqueous solution was then acidified and the liberated acids were collected as already described.

THE PHENOL

The phenolic substance isolated from the active fraction was a light reddish-yellow resin which could not be made to crystallize. It decomposed when an attempt was made to distill it in a vacuum of 3 mm. Four doses of it, each equivalent to 4 pounds of dried plant per hundredweight of animal, were given to sheep 782 without effect. A month later 17 doses of the same quantity were given to this sheep without effect. The sheep also received six doses, total-

ing 28 pounds' equivalent of the phenol, from another lot without effect. Sheep 5 received four doses of 8 pounds' equivalent from a third lot without effect. From these feedings it was apparent that the phenolic constituent of the active fraction is not the poisonous agent in the plant.

THE ACID CONSTITUENTS

The acid constituents were collected, washed free from mineral acid, and dissolved in aqueous sodium carbonate for feeding tests. Sheep 5 received five doses of 4 pounds' and one of 3 pounds' equivalent per hundredweight of animal without effect. The fatty acid constituents of the plant also had been administered to sheep in such extracts as fraction AAA and AABA which were not toxic.

THE PETROLEUM-ETHER PRECIPITATE

In the purification of tremetol the ethereal solution of the substance was mixed with 4 volumes of petroleum ether which occasioned a precipitate of resinous matter. This fraction was redissolved in ether and reprecipitated by petroleum ether, freed from solvent, and tested. In 1925 sheep 5 received 11 doses, each equivalent to 7.5 pounds of dried plant per hundredweight of animal, without effect. These experiments made it clear that the active substance is not acid or phenolic in nature and that it is soluble in the ether-petroleum ether mixture referred to.

VACUUM DISTILLATION OF THE ACTIVE FRACTION

Two separate lots of the active fraction were submitted to distillation under reduced pressure of from 3 to 16 mm. There was extensive decomposition, and a large amount of resinous residue remained in the distilling flask. The main fractions of distillate were united and redistilled. The fraction that came over at 175°–188° C. under 1 mm. consisted of a white solid that was recrystallized from alcohol and then melted at 136°–137°. Analyses gave C, 70.78 and 70.88 per cent; H, 6.35, 6.34, and 5.96 per cent. The vacuum distillates and the residue were administered to sheep but did not affect them. It was evident from the character of the distillates that extensive decomposition of the active material had taken place. It may be noted here that prominent among the decomposition products were acetic acid and a terpenelike substance.

THE ISOLATION OF TREMETOL

It was necessary to prepare a larger quantity of the active fraction in order to complete the study of the plant. From a recently collected lot there was obtained a light-yellow oil that resembled the tremetol obtained from *Eupatorium urticaefolium*. This was subjected to analysis and the figures for carbon and hydrogen agreed with those obtained in the case of the former plant. The fraction was then purified until the molecular weight, determined by the freezing-point method with acetic acid as solvent, was constant. The fraction was identified as tremetol by the following characters: Percentage of carbon and hydrogen; molecular weight (found 262,258, calculated 262.17); optical activity, levorotatory, $[\alpha]_D^{30°} = -33.82°$; and the color reaction with sulphuric acid was positive.

Tremetol is known to cause the typical symptoms of trembles(*4*). Its presence in rayless goldenrod is sufficient to account for the cases of range poisoning caused by the plant. No other constituent of the plant was found toxic to sheep, and all of them, with the exception of the volatile oil, were given in sufficient doses to determine their lack of toxicity. The volatile oil is still being studied.

LOSS OF TOXICITY IN DRIED PLANT

One of the most apparent differences between richweed and rayless goldenrod is the fact that the latter is poisonous when dried as well as when green, whereas richweed rapidly loses its toxicity after it is gathered and when dried is incapable of causing trembles, although it may still be poisonous because of its content of toxic resin acid.

The field experiments showed that rayless goldenrod did not lose much of its toxicity on drying and storing for short periods (*8*). However, the chemical study has shown that rayless goldenrod loses tremetol gradually in storage although at a much slower rate than does richweed. Part of a lot of the former plant extracted 30 months after collection showed a diminished toxicity (sheep 4). Another part of the same lot extracted 55 months after collection contained no tremetol. The extract was worked up to the tremetol fraction and this was administered to sheep 8. Nine doses, equivalent to a total of 60 pounds of dried plant per hundredweight of animal, were given to the sheep. Daily samples of blood were drawn from the left jugular vein. These were tested for the presence of acetone and the amount of blood sugar was determined. The animal showed no effect from the feeding. The fraction fed was later tested for tremetol and that substance was found to be absent. It appeared, therefore, that the plant had lost its toxicity during the four and a half years that had elapsed after its collection.

THE OCCURRENCE OF KETOSIS IN TREMBLES

The appearance of symptoms of ketosis in cases of trembles and milk sickness has been noted especially in richweed poisoning (*1*). Jordan and Harris (*6*) published the report of Woodyat, who determined the presence of acetone in urine from milk-sickness patients. In the cases of rayless-goldenrod poisoning that have come under the observation of the writer, ketosis has been an almost constant complication. Acetone has been detected in the exhaled air and in the urine and blood of sick animals and at post-mortem examination. Carcasses of animals dead of the disease often carried a pronounced odor of the ketone. As with richweed poisoning, however, this symptom is not always present and, when absent, the prognosis is more favorable.

SUMMARY

Rayless goldenrod or jimmy weed (*Aplopappus heterophyllus*) causes trembles, known locally as alkali disease, in certain sections of western Texas, New Mexico, and Arizona. The disease as it occurs in these places is clinically indistinguishable from trembles that occurs in the Central States, although in the latter locality the causative plant is richweed or white snakeroot (*Eupatorium urticaefolium*).

Tremetol, $C_{16}H_{22}O_3$, is the toxic constitutent of rayless goldenrod. This compound was first isolated from richweed, and is the substance in richweed that causes trembles in animals and milk-sickness in man.

Dried rayless goldenrod appears to lose its toxicity slowly. The process is much less rapid than is the case with richweed.

Animals poisoned on rayless goldenrod develop a ketosis, excrete acetone, become hyperglucemic, and in all respects resemble those poisoned on richweed.

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METHODS USED IN TESTING MATERIALS AS REPELLENTS AGAINST THE JAPANESE BEETLE [1]

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INTRODUCTION

Lead-arsenate sprays are recommended by the Japanese beetle laboratory[3] for the protection of late apples, late peaches, shade trees, and foliage of other plants from the attack of the Japanese beetle *Popillia japonica*. There are, however, several favored food plants of the beetle on which lead arsenate can not be used to advantage. Early peaches are very liable to attack by the beetle, but it is inadvisable to spray them with anything that leaves a toxic residue, since, because of the nature of the fruit, the residue can not be removed at the time of harvest. This applies also to early apples, although a certain amount of the residue can be removed from apples by subsequent treatment. The blossoms of flowering plants are severely attacked by the beetle, but a spray which protects by residue alone is effective for only a very brief period, and additional applications are necessary because the leaf and blossom areas are constantly expanding. Various small fruits are attacked by the beetles, and it does not appear at present that a spray or dust could be applied advantageously to such plants. A satisfactory repellent would do much to reduce this type of injury.

The methods described in this paper have been employed in testing over 430 different materials and combinations. As a result of these tests, some materials have been found to possess a definite repellence to the Japanese beetle, and others have been found to be repellent to the beetle only when a certain concentration of their vapor is constantly maintained. Although the problem of finding a satisfactory repellent for the Japanese beetle has not been solved, the methods employed in these tests may be of value to workers engaged on similar problems.

The problem of discovering a material repellent to the Japanese beetle was first undertaken in 1922 at the Japanese beetle laboratory by Loren B. Smith, assisted by T. H. Frison. Since then Mr. Smith has directed the project. The investigations were conducted during 1923 by F. J. Brinley, and during 1924, 1925, and 1926 by E. Avery Richmond. The investigations during 1927 and 1928 were conducted by the writer under the supervision of E. R. Van Leeuwen. As new methods were used in 1927 and 1928, this paper includes only the work done in these years.

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[2] The writer expresses his appreciation of assistance by L. B. Smith, principal entomologist in charge of the Japanese beetle laboratory, E. R. Van Leeuwen, associate entomologist, E. Avery Richmond, D. H Grant, and P. A. Van der Meulen, agents, Bureau of Entomology, and I. L. Hunt, jr., temporary assistant.

[3] SMITH, L. B. JAPANESE BEETLE CONTROL. N. J. Dept. Agr., Bur. Statis. and Insp. Circ. 90, 31 p. illus. 1925.

LOCATION

The cage tests were conducted in an insectary, and a small number of vaporizer tests were conducted in an apple orchard. The greater part of the work was done at Moorestown, N. J., in a neglected peach orchard, which contains 906 trees, 223 of an early variety and 683 of a later variety. The orchard itself is bounded on three sides by uncultivated fields and on the fourth by an abandoned apple orchard. During the summers of 1927 and 1928 heavy crops of early peaches were almost entirely consumed by beetles as soon as they approached ripeness. In 1927 a heavy crop of late peaches, none of which were injured by the beetle, was ready for harvesting about September 12. There was no crop of late fruit the following year. The entire orchard was heavily infested with beetles during the summers of both years, although the infestation was heavier on the early fruit. Field experiments were begun on July 5, 1927, and concluded August 31. In 1928 field work was begun July 9 and ended August 23, because of diminished infestation toward the close of the season.

METHODS

1. Testing material in comparison with a known attrahent.
   A. In traps.
   B. In 6-ounce tins.
      a. Half-hourly observations.
      b. Continuous observation.
2. Spraying or dusting entire trees.
3. Cage tests.
4. Vaporizer tests.
5. Tests with vapor-dispensing devices.

In testing a large number of substances, of which only a few are likely to prove of value, it is advisable to employ a method that will give reliable preliminary results with a small quantity of material. Since volatile and nonvolatile materials could not be tested by the same method, it was necessary to have one for each group. Method 1 was used in testing materials that were volatile, and method 3 was employed for substances which were practically nonvolatile.

After a material has given favorable results in preliminary tests, it should be tested on a larger scale. Such tests were conducted with volatile materials by methods 4 and 5, while nonvolatile materials were tested by method 2. Method 2 was also used in preliminary tests of nonvolatile substances which could be obtained in sufficient quantity for a thorough application to an entire peach tree.

One ounce of a given chemical is sufficient for a complete test by method 1 or 3. Method 4 requires half a pound, method 2 at least a pound, and method 5 several pounds.

METHOD 1.—TESTING MATERIAL IN COMPARISON WITH A KNOWN ATTRAHENT

This method is based upon the use of the attrahents geraniol and eugenol [4] in comparison with the material being tested to ascertain whether or not the test material decreases the attraction of the geraniol-eugenol combination. In all tests geraniol [5] and eugenol were combined with a carrier composed of the following ingredients:

[4] RICHMOND, E. A. OLFACTORY RESPONSE OF THE JAPANESE BEETLE. (POPILLIA JAPONICA NEWM.) Ent. Soc. Wash., Proc. 29: 36–44. 1927.

[5] The geraniol used in all tests was purchased in one lot, the actual purity of which was found by analysis to be approximately 85 per cent.

Bran, 50 gms.; molasses, 26 c. c.; glycerine, 4 c. c.; water, 9 c. c.; and sodium benzoate, 0.1 gm. This carrier was selected by Richmond from among several combinations because it was the most satisfactory for volatile materials in field tests.

Method 1 appears to give a reliable preliminary indication of the value of a material as a repellent, but such an indication is not always borne out when the material is tested on a large scale. Method 1 also makes possible a large number of tests in a comparatively short time and with very small quantities of material.

Material Tested in Traps

Standard traps [6] were used principally to substantiate conclusions based on results obtained by the use of 6-ounce tins. These traps had never been used in this connection before, and it was desired to ascertain how useful they would be and if it would be feasible to use them in place of tins. Each material was tested in triplicate. In each series there were three traps containing geraniol together with the material being tested, and three check traps containing geraniol alone. Twenty-five gms. of the carrier, combined with 2.5 gms. of geraniol and 0.25 gm. of eugenol, was placed in one-half of the bait container of each trap. Twenty-five gms. of the carrier, combined with 5 gms. of the material being tested, was placed in the other half of each bait container in three of the traps.

FIGURE 1.—Standard traps used for testing repellents in the field

Posts were set in the ground parallel to rows of peach trees at a distance of 3 feet from the outer branches. All posts were similarly placed with respect to the trees. A trap containing geraniol was hung from one end of a 3-foot cross arm on the top of the post, and one which contained geraniol together with the material being tested was hung from the opposite end. The six traps in each series were located on adjacent posts. Trapped beetles were collected daily. Figure 1 shows the traps as they were set up in the field.

Material Tested in 6-Ounce Tins

HALF-HOURLY OBSERVATIONS

The 6-ounce salve tins used in these tests were similar to the tins that had been used for several years in the tests of attrahents [7]

[6] RICHMOND, E. A., and METZGER, F. W. A TRAP FOR THE JAPANESE BEETLE. Jour. Econ. Ent. 22: 299-314, illus. 1929.

[7] See footnote 4.

at the Japanese beetle laboratory. Two tins were lashed firmly together side by side to form a double bait can. One tin was filled with 90 gms. of the bran carrier in which 2.5 gms. of geraniol and 0.25 gm. of eugenol were mixed. The other tin was filled with the same weight of the bran carrier to which 5 gms. of the material being tested was added. Each double bait can therefore contained an attractive element, the geraniol-eugenol bait, which was compared with the element being tested. A second double bait can which contained the attractive bait in one tin and the bran carrier alone in the other was used as a check.

Tests were conducted in series. Each series contained three double bait cans treated with different materials, and one check, all of which were hung on one peach tree at equal distances around the periphery and at a height of 4 feet from the ground. All materials were tested in duplicate and the duplicate cans were hung on different trees. Each test was continued until a minimum of 25 beetles had been observed on the check.

The position of each can was changed half hourly according to a prearranged plan. Each of the four positions on a tree was designated by a number, and a tag bearing the number of each position was placed at the proper point. The can which was placed at position 1 was moved to position 2 at the time of the following observation and so on. Nine records were taken daily at half-hourly intervals, from 11 a. m. to 3.30 p. m. At each observation the number of beetles actually present on the cans was recorded.

Results were computed by comparing the number of beetles observed on the treated cans with the number on the respective checks. It was found that when four check cans were placed on the same tree and moved as described above, each attracted approximately the same number of beetles. As the maximum variation was only 12.6 per cent, the results were considered to be sufficiently accurate to serve as a basis for comparison.

CONTINUOUS OBSERVATION

Continuous observations, instead of half-hourly observations, were made during the summer of 1928, and gave better and quicker results.[8] Otherwise the method was the same, except that a check was prepared for each treated can, and that the cans were hung on stands. One treated can and one check were hung from opposite ends of the cross arm of a stand. (Fig. 2.) They were approximately 3 feet apart and 3 feet from the ground. This distance between cans was selected because it was sufficient to prevent any of the odor of the material being tested from reaching the check can, and the height was determined by considerations of convenience. The stand was placed so that each double bait can was approximately 1 foot from the outer foliage of an infested tree. The preference of the beetle for sunlight was met by placing the cans in the sun. That the odor from each pair of cans should be carried directly into the infested tree was assured by placing them on the windward side. The distance of 1 foot from the outer foliage was considered short enough to allow the full odor to reach the infested foliage, yet long enough

[8] This method was considered by Richmond in 1926. (Unpublished notes.)

to compel the beetles to fly in order to reach the cans, thus indicating a definite response.

The beetles alighting on each double bait can were counted during a 10-minute period. The position of the cans on the stand was then reversed to equalize, as far as possible, any variation due to the respective positions of the cans. All beetles were then removed and a second 10-minute count made. The 10-minute period was believed to be long enough to allow the influence of the odor to be felt, yet short enough to permit a reversal of position and a second count before any marked change either in the weather or the intensity of infestation on the tree could occur. This was continued until approximately 50 beetles were recorded on the check cans. Less than 50 beetles was considered insufficient for an accurate indication, and more than 50 unnecessary.

Each material was tested three times, 24 hours being allowed to elapse between tests, so that a series of results under varying conditions might be obtained.

FIGURE 2.—Double bait cans suspended from standard

RESULTS OF COMPARISONS WITH KNOWN ATTRAHENTS

It should be clearly understood that this method does not give results in terms of direct repellence. It merely shows to what extent a chemical will increase or decrease the attraction of geraniol and eugenol when the material being tested is placed in close proximity to the attrahents.

The effect of the material being tested on the attractiveness of the geraniol-eugenol combination may be represented as a quantity, M, derived by the formula:

$$M = \frac{\text{Total number of beetles on treated can or trap} \times 100}{\text{Total number of beetles on check can or trap}}$$

If M equals 0, it indicates that the effect of the material being tested completely counteracted the attraction of geraniol. If M equals more than 100, it indicates that the material being tested increased the attraction of geraniol.

Three hundred and six different materials were tested by this method, which involved 1,086 tests. The following 45 materials had values for M of less than 25 in all cases and include all those substances which might be expected to repel beetles when used alone. The materials are arranged according to the degree by which they decreased the attraction of the geraniol-eugenol combination, o-cresol being superior to all others in this respect:

O-cresol, pine-tar oil, phenol, Dippel's oil, high-boiling tar acids (Welsh), coal-tar neutral hydrocarbon oil, trichlorobenzene, crude dichlorobenzene No. 1, alpha chloronaphthalene, crude dichlorobenzene No. 2, white creosote from coal tar, destructively distilled pine wood oil, methyl anthranilate, xylidine, proprietary disinfectant No. 1, guaiacol, crude coal-tar creosote, methyl salicylate, carvacrol, cyclohexyl acetate, p-bromophenol, dimethyl aniline, p-chlorophenol, discard oil (from Dippel's oil), naphthalene, quinoline, ethyl chloroacetate, o-bromophenol, quinaldine, hyssop oil, alpha naphthol, creosote carbonate, ethyl benzoate, furfuryl alcohol, vanillin, o-chlorophenol, furfural and pine-tar oil mixture, acetaldehyde ammonia, cresylic acid (Welsh), cade oil, Carolina heavy pine tar oil, safrol, cajeput oil, acetone, and cascarilla oil.

A rough classification of these materials is given in Table 1. This table also gives the total number of substances in each group that were tested, and the number and percentage of those having a value under 25 for M, together with the number and percentage of those of each group having values over 100. The limitations of such an arrangement are obvious, since the composition of many of the substances is complex and incompletely known. The classification is included only to illustrate certain facts regarding the value of method 1.

TABLE 1.—*Classification of materials having values of 25 or under and of 100 or over for M*

Chemical group [1]	Materials tested	Materials having M value under 25		Materials having M value over 100	
	Number	*Number*	*Per cent*	*Number*	*Per cent*
Acids, organic	5	0	0	3	60.0
Alcohols	22	1	4.5	5	22.7
Aldehydes	19	2	10.5	3	15.8
Amino compounds	11	4	36.4	2	18.2
Balsams, oleoresins	5	0	0	1	20.0
Coal-tar creosotes	7	7	100.0	0	0
Cyclic nitrogen compounds	5	2	40.0	1	20.0
Empyreumatic oils	11	7	63.6	0	0
Essential oils	88	3	3.4	15	17.0
Esters	41	6	14.6	12	29.2
Ethers	20	3	15.0	3	15.0
Fatty oils	3	0	0	2	66.6
Halogenated organic compounds	40	10	25.0	1	2.5
Hydrocarbons	20	2	10.0	1	5.0
Ketones	17	2	11.8	6	35.3
Nitro compounds	1	0	0	0	0
Phenols	20	12	60.0	1	5.0
Plant extracts and juices	7	0	0	4	57.1
Sulphur compounds	8	0	0	1	12.5

[1] A number of chemicals possess the functions of 2 or more groups and are included in each, thus causing duplication.

It will be seen in the table that the coal-tar creosotes and empyreumatic oils, as groups, had the lowest M values. In all the coal-tar creosotes tested, the value of M fell below 25, and 7 of the 11 empyreumatic oils had values under that figure. The phenols and cyclic nitrogen compounds had the next higher values in this group. On the other hand, only a small percentage of the materials in certain other groups, such as essential oils, esters, and ketones, had values under 25, while a much greater percentage had M values of over 100.

The purpose of the preliminary tests was to obtain an indication of the value of a large number of materials in as short a time as possible. The method under discussion met this qualification and has proved especially valuable in the search for a repellent for the Japanese beetle.

The trap method and the continuous observation method are far superior to half-hourly observations and are equally effective.

In a number of cases, certain substances have greatly reduced the attractive power of geraniol when tested by method 1, but when used in spray, dust, or vapor form, on or near flowering plants, have afforded little or no protection from beetle attacks. As the concentration of vapor was known to be less when the material was used on a large scale than when it was used in comparison with geraniol and eugenol in 6-ounce tins or traps, it appears that satisfactory results with volatile materials can be obtained only by maintaining a concentration of repellent vapor which is comparable to that which was obtained in the trap or double bait-can tests.

METHOD 2.—SPRAYING OR DUSTING ENTIRE TREES [1]

Two peach trees of approximately the same size, and located in the same part of the orchard, were used for each test in 1927. There was sufficient space between them to prevent a material applied on one from affecting the beetle infestation on the other, and trees were chosen on which the beetle infestation was approximately the same. One of the trees was treated with the material being tested, and the other was sprayed with water only to serve as a check. The beetles on each tree were counted before and after spraying. Treatments were made as soon as the beetles became active in the morning, usually between 9 and 10 o'clock. Counts of the number of beetles present on each tree were made at 15-minute intervals throughout the remainder of the day until 3 p. m. If a material still appeared to be repellent at the end of the first day, a single observation was made each day thereafter until no repellence was evident.

Ratios for the treated and check trees were obtained by dividing the average infestation computed from the 15-minute counts by the original number of beetles present on the tree before the test was begun. The repellence for each material was considered to be the difference between the ratio for the treated tree and the check.

In 1928 a large number of the sprays and dusts were applied to early peach trees, which bore a fair crop of fruit. Treatments were made before any beetle injury was noted on either fruit or foliage. Several materials were applied after the trees were infested, and a number of tests were begun after the fruit was gone. Check trees were left untreated when no beetle injury was noted, but in the tests started after the insects became numerous the check trees were sprayed with water alone. Ten tests were usually begun at one time, and two checks, together with a tree dusted with 1 to 9 lead arsenate and hydrated lime, were included in each series.

If a tree was infested with beetles when it was treated, a count was made of the number before and after the treatment was applied. Daily observations were made on all treated and check trees as long as a material showed any repellence, or as long as was necessary in order to obtain an indication of the value of the material.

[1] This method is a modification of that used by Richmond prior to 1927.

Almost all the materials tested during 1928 were nonvolatile or nearly so. The purpose of these tests was to ascertain the repellence of residues of varying conspicuousness. Some materials were hardly visible after application, whereas others left a very conspicuous coating on the foliage.

This method was employed as the final step in the determination of the repellent value of materials in spray or dust form. As the procedure is similar to that followed in practical control work, definite and final conclusions may be made as to the particular value of each material tested. Some of the results are given to illustrate the value of this method.

Results of Spraying or Dusting Entire Trees

Resins, oleoresins, and balsams

Resins, oleoresins, and balsams were emulsified and used at the rate of 1 part of the actual material to 128 parts of water. Wheat flour was added as a spreader at the rate of 2 pounds to 50 gallons. All the materials adhered well to the foliage and left only a slightly conspicuous residue.

Fifteen materials applied before infestation on early peach trees which were bearing fruit delayed the infestation of the fruit from one to four days and gave considerable protection to the foliage as compared with the fruit and foliage injury on the untreated checks. The fruit was not protected for a sufficient length of time for the treatments to be of practical value. Included in this group of materials and listed in the order of decreasing repellence, are the following: Gamboge, labdanum, olibanum, orris root, and scammony resins, Canada fir balsam, elemi resin, tonka oleoresin, styrax (American), ammoniac, benzoin (Siam), and styrax (Asiatic) resins, male fern oleoresin, and myrrh resin. Five other substances of this nature had practically no effect in preventing infestation either of fruit or of foliage.

Guaiac resin, pine-needle extract (German), and Peru balsam were applied to infested trees bearing fruit. These materials decreased the infestation on the foliage of the trees to which they were applied but afforded no protection to the fruit, which was consumed almost as soon as that of the check trees. Cubeb oleoresin, which was applied at the same time, failed to give any protection to fruit or foliage.

Oleoresin of black pepper was the only material of this group that was tested on an infested tree on which there was no fruit. It gave excellent protection to the foliage.

Pharmaceutical plant extracts

Commercial United States Pharmacopoeia or National Formulary extracts of drug plants were used at the rate of 1 part to 64 parts of water, with 0.2 part of a 10 per cent saponin emulsifier. Flour was added as a spreader at the rate of 2 pounds to 50 gallons of water, and fish oil was added as a sticker at the rate of 1 part to 500 parts of water.

Eleven materials were applied on uninfested trees which were bearing fruit, 8 on infested trees with fruit, and 49 on infested trees without fruit. None of these materials retarded or decreased beetle infestation on fruit or foliage, as compared with untreated checks.

The results indicate that certain materials of a resinous nature, which leave little perceptible residue but which stick well to fruit and foliage of peach trees, have much better repellent properties than any of the pharmaceutical extracts which were tested at the concentrations given above.

METHOD 3.—CAGE TESTS

The value of certain materials as repellents for the Japanese beetle was tested under cage conditions during the summer of 1927. Volatile materials were used almost exclusively, and the results were unsatisfactory. Since the cage method has several advantages over field methods it was decided to use it during the summer of 1928, but only in testing nonvolatile materials. Most of the materials tested could not be used in the field because of the small quantity available.

The cages were similar to those used in insecticide studies at the Japanese beetle laboratory for several years. They were 24 inches long, 12 inches wide, and 14 inches high. Bottom and ends were of wood, with a hinged door at one end to permit the introduction of insects and plants. (Fig. 3.)

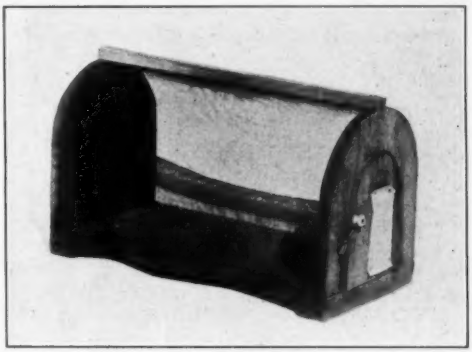

FIGURE 3.—Cage employed to test repellents by method 3

All materials were sprayed or dusted on newly collected peach twigs, which were kept in a fresh condition by placing them in 2-ounce bottles of water held upright on the floor of the cage by brackets. Fifty beetles were used in each cage. Each experiment lasted four days, unless most of the beetles died sooner, and observations were made every 24 hours.

When a plant in the field has been treated with a material repellent to the beetle, the insect merely ignores it and selects an untreated plant. In order to approximate field conditions, treated and untreated twigs were used in the same cage, and the degree of repellence was determined from the number of beetles feeding on the treated twigs. As a further test for each material, treated food was used alone in other cages to ascertain whether beetles would feed on it when no untreated food was available.

For each material there were 2 cages containing both treated and untreated twigs and 2 cages containing treated twigs only. Tests were made in series, each of which consisted of five groups of 4 cages each, treated as described above, one group of 4 cages containing twigs dusted with lead arsenate and hydrated lime (1 to 9), and 2 cages containing untreated twigs to serve as checks.

All materials tested by this method are practically nonvolatile, and any repellence must have been caused by the presence of a residue. Deposits left on the foliage by the different treatments were of varying conspicuousness.

The result of each test was obtained by comparing the total number of beetles feeding on the treated twigs during the entire course of the

test with the number feeding on the respective checks. The effect of the material in repelling beetles may be represented as a quantity, M or N, derived as follows:

$$M = \frac{\text{Number of beetles feeding on treated twigs} \times 100 \text{ (in cages containing both treated and untreated twigs)}}{\text{Number of beetles feeding on untreated checks}}$$

$$N = \frac{\text{Number of beetles feeding on treated twigs} \times 100 \text{ (in cages containing treated twigs only)}}{\text{Number of beetles feeding on untreated checks}}$$

Results of Cage Tests

When the value of M fell below 25, the material was considered to be repellent to the beetles. When the value of N fell below 25, the repellence was considered to be still greater. A good example is afforded by the lead arsenate-hydrated lime treatments. In 15 tests the average value for M was 17, and that for N was 19, which indicates clearly that this dust was strongly repellent to the beetle. Numerous applications of this dust in the field have also demonstrated its value as a repellent. Since the results from its use in the cages were similar to those in the field, it appears that data obtained by this method are a good indication of similar results in the field.

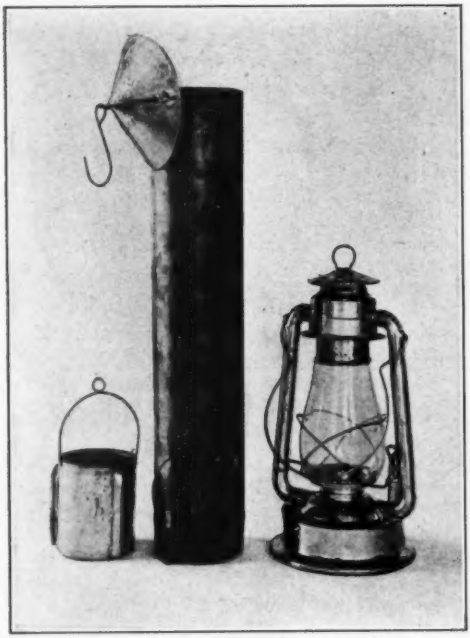

FIGURE 4.—Vaporizer (unassembled) used in testing repellents by method 4

When the value of M was less than 25 and the value of N was greater, it was considered as an indication that the material would probably be repellent if abundant untreated foliage were available, but that it would not prevent feeding if the beetles were hungry.

If the values of both M and N are greater than 25, the material has little or no repellent value.

METHOD 4.—VAPORIZER TESTS

Vaporizers were used for applying constant heat to certain chemicals to increase the rapidity of volatilization. The vaporizer[10] which

[10] This apparatus was devised by P. A. Van der Meulen and F. E. Mehrhof, agents, Japanese beetle laboratory.

proved most satisfactory under field conditions (fig. 4) consisted of a 30-inch length of 5-inch stovepipe and a lantern with its chimney. The stovepipe was fitted over the top of the lantern so as to cover all but the lower portion of the chimney and was fastened to the lantern with heavy wire. As the flame of the lantern was not exposed to air currents, there was no danger of its being blown out. The reservoir contained sufficient oil for burning 24 hours. The chemical being tested was placed in a can which was held in the end of the stovepipe by three friction fins and could be raised or lowered by means of a heavy wire bail. A cone of galvanized iron was attached 1 inch above the top of the stovepipe to protect the chemical from rain. The vaporizer was suspended from a heavy wire hook above the cone.

FIGURE 5.—Vaporizer shown in Figure 4, in operation

The vaporizers were placed in the orchard between 9 and 10 a. m., after the beetles had become active at a place where there was a heavy beetle infestation, so that if the material proved to be repellent, the action would be particularly noticeable. They were taken in at 3 p. m. When used in the orchard the vaporizer was placed in the outer branches to windward of the tree, with the top usually 4 to 5 feet from the ground. In several tests the vaporizers were placed near clumps of evening primrose and rows of marigolds, which grew to a height of 3 to 5 feet. The apparatus was then suspended from a 4-foot standard (fig. 5) on the windward side of the plants, with the top of the vaporizer slightly below the highest shoots.

The apple trees were very large, and the vapor could not reach the entire leaf area. The effect was, therefore, more or less localized. As the peach trees were smaller, the vapor could easily reach all parts of the tree. The infestation was very heavy on both apple and peach trees, and, as an accurate count of the beetles was impossible, the only indication of the repellence of a material was a marked decrease in the infestation.

RESULTS OF VAPORIZER TESTS

Thirteen materials were tested by this method, and each was used on at least two different occasions. Eight substances, carvacrol, o-chlorophenol, coal-tar neutral hydrocarbon oil, o-cresol, Dippel's oil, methyl anthranilate, pine-tar oil, and quinoline, reduced the infestation by at least 75 per cent in each test. All of these materials

greatly reduced the attraction of geraniol and eugenol in tests by method 1. Only one substance (a crude chloronaphthalene) which had a value for M of less than 25 in method 1 failed to show marked repellence when tested in a vaporizer.

The results obtained by the use of this apparatus indicate that the method is a good one, and, for the first time since the project was organized, it appears possible to maintain effective repellence by means of a volatile material.

METHOD 5.—VAPOR-DISPENSING DEVICES

Liquid-dispensing bottles (fig. 6), designed by P. A. Van der Meulen, were used during the summer of 1927. Each bottle was suspended from a peach tree, care being taken to have the apparatus located as near the center as possible. A tin pan containing a large sponge was hung directly under the bottle so that the liquid falling from the bottle would be caught on the sponge. A tree in the immediate vicinity of each tree bearing a bottle was used as a check. The trees were of an early variety. The fruit was approaching ripeness, and fruit and foliage were heavily infested with beetles when the tests were begun. None of the materials gave satisfactory results when tested in this apparatus.

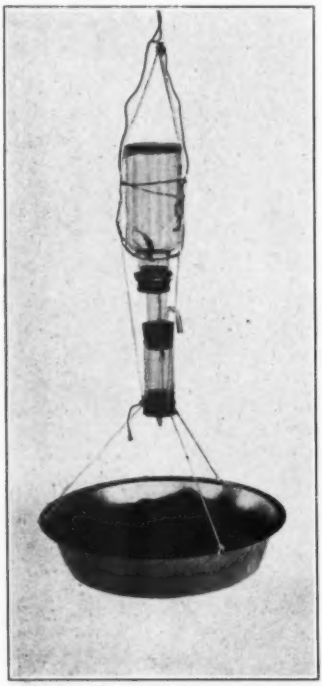

FIGURE 6.—Liquid-dispensing bottle used to test repellents by method 5

During 1928 each material was tested on two peach trees bearing fruit, and all tests were begun before the trees were infested. Solid materials were mixed with bran or sawdust and broadcast on the ground under a tree. They were also placed in cylindrical 16-mesh wire-screen baskets of 1-pound capacity and suspended from the branches. Five baskets were used for each tree, and were distributed about the tree at a height of 5 to 6 feet, well in toward the trunk. Liquid materials were placed in dispensing bottles, or mixed with bran and broadcast under the trees, or absorbed in balls of cheap cotton batting.[11] Five of these balls were suspended from each tree in the same manner as the wire baskets. Two dispensing bottles were used for each tree, and were located several feet apart on opposite sides of the trunk at a height of 5 to 6 feet.

Treatments were repeated at weekly intervals, or more often if the odor could not be noticed around the tree. The average quantity of each chemical was 5 pounds per treated tree. Each chemical was used both on the ground under the tree and in the foliage, and two trees were used in each test.

[11] Cotton balls were first employed by Richmond in 1926.

Results of Tests with Vapor-Dispensing Devices

Six of the materials tested, o-cresol, Dippel's oil, alpha naphthylamine, phenol, pine-tar oil, and quinoline, afforded excellent protection to peach foliage and retarded the infestation of fruit from one to three days, as compared with the untreated checks. Five of these substances had a value of less than 25 for M when tested by method 1. Alpha naphthylamine had a value of 37. The results of the tests with vapor-dispensing devices are a further check on the accuracy of method 1.

While the vapor of the various materials was sufficiently strong to protect foliage, it did not prevent infestation of the fruit, which took place almost as soon as it did on the check trees. This method provides a means for using volatile materials in other than spray or dust form but can be employed only with comparatively cheap substances.

SUMMARY

The five methods discussed in this paper were used during 1927 and 1928 in testing 430 materials, alone and in combination, as repellents for the Japanese beetle. More than 1,500 tests were conducted.

Tests of volatile materials in comparison with known attrahents, as conducted by method 1, give indication of the probable value of such substances as repellents in a comparatively short time, and with the use of a small quantity of each material. This method indicates, further, that certain groups of chemicals are much more likely to be repellent to the beetle than others.

The use of method 2, in which entire trees are sprayed or dusted, presents a means for testing volatile and nonvolatile materials on a scale comparable to that of practical control operations.

Method 3 makes it possible to ascertain the value of a nonvolatile material as a repellent under cage conditions with a minimum expenditure of time and material.

Vaporizers, used in method 4, furnish a useful means for increasing the vapor concentration of volatile materials and of testing the value of such substances as repellents.

A number of miscellaneous vapor-dispensing devices, employed in method 5, failed to give satisfactory results when used with certain materials.

THE PROPERTIES OF ARACHIN AND CONARACHIN AND THE PROPORTIONATE OCCURRENCE OF THESE PROTEINS IN THE PEANUT [1] [2]

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INTRODUCTION

The importance of peanuts both as an article of human food and as a feedstuff is becoming generally recognized. The high nutritive value of the proteins of peanuts has been shown in previous publications from this laboratory, and elsewhere. Peanut press cake has been shown to be an excellent protein concentrate to supplement rations containing proteins deficient in essential amino acids.

Knowledge regarding the proteins of the peanut is limited almost entirely to that contained in an early publication by Ritthausen (*6*),[3] and in a more recent series of articles from the Bureau of Chemistry and Soils of the United States Department of Agriculture. Ritthausen extracted oil-free peanut meal with various saline and alkaline solutions and precipitated the globulin fraction by dilution with water, and by acidification with acetic and sulphuric acids. No evidence was obtained indicating the presence of more than one globulin. In the investigations carried on in the Bureau of Chemistry, Johns and Jones (*1*), in 1916, isolated from peanut meal two globulins which they named arachin and conarachin. The arachin was precipitated from a 10 per cent sodium chloride extract of the meal (*a*) by dilution with water, (*b*) by making the salt extract 20 per cent saturated with ammonium sulphate, and (*c*) by saturating the salt extract with carbon dioxide. The more soluble conarachin was precipitated by dialyzing the filtrate from the arachin, or by saturating it with ammonium sulphate. Arachin was obtained in much the larger quantity and different from conarachin in having only about one-third as much sulphur, and in containing less basic nitrogen. Besides arachin and conarachin a trace of a substance having the properties of an albumin was isolated. The quantity, however, was so small that its nature could not be definitely established. No evidence was obtained indicating either the presence or the absence of proteins of the prolamin or glutelin type. Later, studies of the chemical composition of arachin and conarachin were made, including a study of the distribution of the nitrogen by the Van Slyke method (*2*), the determination of certain amino acids by colorimetric methods (*4*), and the hydrolysis of arachin with subsequent determination of the resulting amino acids by the Emil Fischer ester method (*3*).

In these studies attention was confined chiefly to the chemical character of the two globulins. There were no quantitative data

[1] Received for publication Dec. 31, 1929; issued April, 1930.

[2] Constructed from a dissertation presented by Millard J. Horn in June, 1929, to the faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of doctor of philosophy.

[3] Reference is made by number (italic) to "Literature cited," p. 682.

regarding the proportion in which arachin and conarachin occur in the peanut. The methods available for the isolation and preparation of proteins in a condition satisfactory for their characterization usually require the sacrifice of yields for the sake of purity. Even though the amino-acid composition of the individual proteins in a given food product is known, it is obviously impossible to estimate the percentages of amino acids in the whole material unless data are available regarding the relative proportions in which the proteins occur. This is an important consideration from a practical standpoint. The dietitian or animal husbandman is chiefly interested in knowing how much of the nutritionally essential amino acids the whole food or feedstuff contains rather than the percentages of amino acids in the individual proteins. The investigation described in this paper was undertaken to obtain more information regarding the physical properties of arachin and conarachin, as well as further evidence showing whether peanuts contain proteins other than globulins, and whether they contain nitrogen of a nonprotein nature.

PREPARATION OF THE MEAL AND EXTRACTION OF THE GLOBULINS

Raw, shelled Virginia peanuts were ground in an electrically driven mill, and most of the oil was removed by two or three extractions with petroleum ether. The meal was again ground as fine as possible, and the residual oil removed by repeated extractions with ethyl ether. The meal thus prepared contained 7.36 per cent of nitrogen, equivalent to 40.48 per cent of protein ($N \times 5.5$, the conversion factor for peanut proteins).[4]

Fifty grams of the meal was shaken for three or four hours in a 500 c. c. centrifuge bottle with 250 c. c. of 10 per cent sodium chloride solution. The suspension was then centrifuged, and the supernatant liquid decanted. The residue was repeatedly extracted in a similar manner with fresh portions of salt solution until no more nitrogen was removed. The extracts were combined, filtered, and diluted to 2 liters with 10 per cent salt solution. This is hereafter referred to in this paper as the extract. For quantitative work on the peanut proteins it is necessary to use freshly prepared extracts. After the saline extracts have stood for about three days, even in an ice box, changes take place, resulting in the precipitation of a light material and the formation of nonprotein nitrogen, which increases in quantity as the extract continues to stand. After a 10 per cent sodium chloride solution of pure arachin had stood in the ice box for several days, it was found that material coagulating at three different temperatures could be separated from the saline solution. The precipitation limits with ammonium sulphate were also changed. With an old solution of arachin a clear-cut separation between arachin and conarachin could not be effected. Small quantities of material separated at 50 per cent, 60 per cent, and 70 per cent of saturation with ammonium sulphate. Nitrogen determinations on aliquots of the extract showed that it contained 86 per cent of the total nitrogen in the meal.

[4] Inasmuch as arachin and conarachin represent more than four-fifths of the total nitrogen in peanuts, and since both contain 18.29 per cent nitrogen, the figure 5.5 is used as the factor for converting the percentage of nitrogen into that of protein.

PRELIMINARY TESTS ON THE EXTRACT

Coagulation tests were made by heating 10 c. c. portions of the extract in a test tube immersed in a water bath, the temperature increasing at the rate of about 4° C. per minute. The first coagulum appeared at 80° C. This was filtered off, and the clear filtrate on further heating became somewhat cloudy at 94°, but no coagulation occurred even when the filtrate was heated to boiling temperature. When the hot solution was slightly acidified with acetic acid, however, a copious precipitate formed.

Precipitation tests with ammonium sulphate likewise showed no evidence of the presence of more than two proteins. Measured quantities of a saturated solution of ammonium sulphate were successively added to 10 c. c. of the extract so as to raise the concentration of ammonium sulphate in the extract by increments of 10 per cent of saturation. After each addition any precipitate formed was filtered off, and another portion of ammonium sulphate added to the clear filtrate. The first appearance of cloudiness occurred at about 20 per cent of saturation, and a gradual increase in the precipitate formed was obtained up to 40 per cent of saturation. Beyond that there was no precipitation until a fraction separated between 70 and 80 per cent of saturation. Continued addition of ammonium sulphate to the filtrate from this fraction up to complete saturation caused no further precipitation. The final stage in completely saturating the filtrate was accomplished by addition of solid ammonium sulphate.

From the results of the coagulation and precipitation tests it was concluded that the extract contained only two proteins.

PROPERTIES OF ARACHIN

When separated from a salt solution by precipitation with ammonium sulphate, or by dilution with water, arachin settles out as a sticky, heavy precipitate. By stirring this precipitate with gradually increasing concentrations of alcohol, and finally dehydrating it with absolute alcohol and ether in the usual way, arachin can be obtained in the form of a fine, white powder. Arachin can also be precipitated from a 10 per cent sodium chloride solution by adding water until a slight cloudiness appears, and then saturating the liquid with carbon dioxide gas.

COAGULATION TEMPERATURE

For the coagulation tests a fresh solution of arachin was prepared as follows: A fresh, filtered, 10 per cent sodium chloride extract of the peanut meal was made 40 per cent saturated with ammonium sulphate. The precipitated arachin was redissolved in salt solution, and reprecipitated by dilution with 10 volumes of water. From half a gram to a gram of the moist precipitate was dissolved in 20 c. c. of 10 per cent sodium chloride solution.

Ten cubic centimeter portions of the arachin solution when slowly heated began to show cloudiness at 90° C., but no precipitate formed even when the solution was boiled. When the hot solution was slightly acidified with acetic acid a heavy precipitate formed.

SPECIFIC ROTATION

For determining the specific rotation of arachin a 10 per cent sodium chloride solution was used. The average of several closely agreeing polarimetric readings gave $[\alpha]\frac{20}{D} = -39.5°$. (Table 1.)

TABLE 1.—*Optical rotation of arachin in 10 per cent sodium chloride solution*

Arachin in solution	Angular rotation	Specific rotation [a]
Grams per c. c.	*Degrees*	*Degrees*
0.0307	−1.2129	−39.5
.00880	−.34657	−39.3
.04085	−1.5595	−38.1
.0205	−.7971	−38.9
.0192	−.7971	−42.5
		Av. −39.5

[a] $[\alpha]\frac{20}{D} = \frac{\alpha}{l \times w}$ where α = specific rotation, l = length of tube, w = weight of protein in grams per cubic centimeter, and α = reading in angular degrees.

PRECIPITATION LIMITS WITH AMMONIUM SULPHATE

Ten cubic centimeters of a 10 per cent sodium chloride solution containing 2 per cent of arachin was treated with trichloracetic acid. The precipitate was filtered off, and the filtrate examined for nitrogen. No nitrogen could be detected. Measured quantities of a saturated solution of ammonium sulphate were then successively added to 10-c. c. portions of the solution. The precipitates were filtered off, and trichloracetic acid was added to the filtrates. The smallest quantity of ammonium sulphate which had to be added so that the filtrate would give no turbidity with trichloracetic acid, thus showing complete precipitation of the arachin, was found to be at 40 per cent of saturation. Most of the arachin was precipitated at 30 to 32 per cent of saturation.

PROPERTIES OF CONARACHIN

Besides the differences in their chemical composition as noted in previous publications (*1*, *2*), the two peanut proteins are distinguished by marked physical differences. Conarachin is much more soluble than arachin. It can not be precipitated from its salt solution by dilution with water without the use of enormously large volumes. It also differs greatly from arachin in its coagulation temperature and precipitation limits with ammonium sulphate and in specific rotation.

COAGULATION TEMPERATURE

Solutions of conarachin were prepared for the coagulation tests as follows: The arachin was first separated from a fresh salt extract of the meal by precipitation with ammonium sulphate (40 per cent of saturation), and the filtrate dialyzed in parchment bags against running water for 20 days, toluene being used as a preservative. The clear dialysate was decanted, and the precipitated conarachin was washed with distilled water and dissolved in 10 per cent sodium chloride solution (1 gm. moist precipitate per 40 c. c. of solvent).

When the conarachin solution was heated slowly the globulin flocculated at 80° C. On being heated to boiling the filtrate remained clear.

SPECIFIC ROTATION

Specific rotation determinations made on 10 per cent sodium chloride solutions of conarachin gave an average of −42.7°. (Table 2.)

TABLE 2.—*Optical rotation of conarachin in 10 per cent sodium chloride solution*

Conarachin in solution	Angular rotation	Specific rotation
Grams per c. c.	*Degrees*	*Degrees*
0.00898	−0.38122	−42.4
.00149	−.19330	−43.0
		Av. −42.7

PRECIPITATION LIMITS WITH AMMONIUM SULPHATE

This determination was similar to that described for arachin. No precipitation occurred on addition of ammonium sulphate until 80 per cent of saturation was reached. All the conarachin was precipitated at 80 to 85 per cent of sautration.

CHARACTER OF THE NITROGEN IN THE PEANUT EXTRACT

Before proceeding with the quantitative determinations of arachin and conarachin it was necessary to study the behavior of the peanut proteins toward certain protein-precipitating agents, in order to ascertain whether all the nitrogen in the extract represented intact protein molecules,[5] or whether the extract also contained proteoses or peptones, or nitrogen of nonprotein character.

Careful addition of a 16 per cent aqueous solution of trichloracetic acid to 10 cubic centimeters of the fresh extract precipitated all but a neglibile quantity of the nitrogen.

BEHAVIOR OF ARACHIN TOWARD TRICHLORACETIC ACID, TANNIC ACID, AND TUNGSTIC ACID

Five grams of pure, freshly prepared arachin was dissolved in 10 per cent sodium chloride solution, and the solution diluted in a volumetric flask with water to 100 c. c. Four 5 c. c. portions of the solution were measured out. One portion was used for the determination of total nitrogen, the second was treated with 15 per cent trichloracetic acid until no furter precipitation occurred, the third was similarly treated with an aqueous 10 per cent solution of tannic acid, and the fourth with a mixture of equal volumes of sodium tungstate and two-thirds normal sulphuric acid. These reagents are commonly used as specific precipitants for intact protein. The resulting precipitates were removed by filtration and washed with 10 per cent sodium chloride solution containing a few drops of the precipitating reagent. Nitrogen was determined in the combined filtrate and washings. The results given in Table 3 show that the three reagents

[5] The term "intact protein molecules" is used to refer to whole, original protein molecules precipitable by trichloracetic acid and tungstic acid, as distinguished from smaller molecules, such as proteoses and peptones that may occur as products of the first stages of degradation of parent protein molecules.

precipitated practically all the intact protein. Five cubic centimeters of the arachin solution contained 0.02527 gm. of nitrogen.

TABLE 3.—*Quantity of arachin precipitated out of 10 per cent sodium chloride solution by various protein precipitants*

[The solution originally contained 0.02527 gm. of nitrogen]

Precipitant	Nitrogen left in the filtrate		Percentage of arachin precipitated
	Grams	Percentage of total nitrogen	
Trichloracetic acid	0.00028	1.11	98.99
Tannic acid	.0002	.79	99.21
Tungstic acid	.0003	1.18	98.82

In order to find out whether any free amino acids might also be precipitated by these reagents along with the protein, precipitation tests were made with arachin in the presence of a mixture of amino acids. The amino acid mixture was prepared by boiling 50 gm. of the α-globulin of tomato seed with a mixture of equal volumes of concentrated hydrochloric acid and water until the hydrolysis was completed, as shown by a negative biuret test. Most of the free hydrochloric acid was removed by concentrating the hydrolysate under reduced pressure to a thick sirup. The residue was dissolved in water, and the solution again concentrated. The final residue was dissolved in water and neutralized with sodium hydroxide.

After the solution was slightly acidified with hydrochloric acid, an equal volume of 95 per cent alcohol was added, followed by the addition of a 10 per cent suspension of calcium hydroxide until all the humin was thrown down, and the solution remained clear. The ammonia was then removed by distilling the mixture under reduced pressure until about half the volume had passed over. The concentrated solution of amino acids was slightly acidified and warmed with charcoal on the steam bath for an hour. The mixture was filtered, and the filtrate diluted with water to 500 c. c.

Portions containing 5 c. c. of the arachin solution and 0.5 c. c. of the amino acid solution (which also contained 10 per cent sodium chloride) were treated with trichloracetic acid, tannic acid, and tungstic acid in the manner described. The precipitates were filtered off and washed, and nitrogen was determined in the filtrates. As shown in Table 4, practically none of the amino acid nitrogen was precipitated with the arachin.

TABLE 4.—*Precipitation of arachin nitrogen from 10 per cent sodium chloride solution by various protein precipitants, in the presence of amino acids*

[Nitrogen in 5 c. c. of arachin solution, 0.0171 gm.; nitrogen in 0.5 c. c. of amino acid mixture, 0.0053 gm.]

Precipitant	Nitrogen left in filtrate	Nitrogen in precipitate *
	Grams	*Grams*
Trichloracetic acid	0.0052	0.0172
Tannic acid	.0057	.0167
Tungstic acid	.0054	.0170

* By difference.

The precipitation of arachin from a solution containing peptone was also studied. A 10 per cent sodium chloride solution of a commercial peptone was prepared. To portions of a mixture containing 5 c. c. of arachin solution and 0.5 c. c. of the peptone solution, trichloracetic acid, tannic acid, and tungstic acid were separately added in the manner described. The precipitates were washed, and nitrogen was determined in the filtrates. As shown in Table 5, trichloracetic acid precipitated all the arachin but none of the peptone. In addition to the arachin nitrogen, 39.13 per cent of the peptone nitrogen was precipitated by tannic acid and 44.66 per cent by the tungstic acid.

TABLE 5.—*Precipitation of arachin nitrogen from a 10 per cent sodium chloride solution by various protein precipitants, in the presence of peptone*

[Nitrogen in 5 c. c. of arachin solution, 0.0171 gm.; nitrogen in 0.5 c. c. of peptone solution, 0.00253 gm.]

Precipitant	Nitrogen left in filtrate
	Grams
Trichloracetic acid	0.00253
Tannic acid	.00154
Tungstic acid	.00140

QUANTITATIVE ESTIMATION OF ARACHIN AND CONARACHIN

The percentage of arachin was determined by three different methods which gave closely agreeing results.

ESTIMATION OF ARACHIN BY PRECIPITATION WITH AMMONIUM SULPHATE

The wide difference in the precipitation limits of arachin and conarachin with ammonium sulphate affords an effective means for their separation.

For the quantitative estimation of arachin 500 c. c. of the fresh, clear filtered sodium chloride extract of peanut meal was placed in a centrifuge bottle, and enough ammonium sulphate added to make the solution 40 per cent saturated. After standing for several hours the mixture was centrifuged. The arachin settled as a viscous, compact layer on the bottom, from which the clear supernatant liquid could be readily decanted. The arachin was again redissolved in 10 per cent sodium chloride solution and reprecipitated with ammonium sulphate in the manner just described. For further purification it was again redissolved in as little 10 per cent salt solution as possible and finally precipitated by dilution with 10 volumes of distilled water. After the whole was centrifuged, the precipitate was dried by first suspending it for 24 hours each in 95 per cent alcohol, and then in absolute alcohol, and finally in absolute ether. The white material was then allowed to stand in a vaccum dessicator over sulphuric acid. Triplicate determinations made in this manner yielded 2.85, 2.84, and 3 gm. of arachin, respectively. Since these quantities were obtained from 500 c. c. aliquots of 2 liters of extract from 50 gm. of meal, they represent, respectively, 22.8, 22.7, and 24 per cent, or an average of 23.2 per cent of the oil-free peanut meal. Even with the exercise of greatest care, some losses occurred during the purification of the arachin. Consequently these figures are somewhat low.

ESTIMATION OF ARACHIN AND CONARACHIN

COAGULATION OF CONARACHIN

Inasmuch as it had been found that, with the exception of a negligible quantity, all the nitrogen in the fresh salt extract of the meal was precipitable with trichloracetic acid, and that practically all the nitrogen in the extract represented arachin and conarachin, for the estimation of arachin and conarachin advantage was taken of the wide range in the temperatures at which these two proteins coagulate. Ten cubic centimeters of the extract was slowly heated to 85° C. The coagulum was removed on a folded filter and well washed with 10 per cent sodium chloride solution. Determination of nitrogen in the filtrate from the coagulated conarachin gave a value equivalent to 2.3 gm. of arachin nitrogen calculated for the 2 liters of extract from 50 gm. of meal. Since arachin contains 18.29 per cent of nitrogen, the foregoing quantities of nitrogen corresponds to 12.5 gm. of arachin, or 25 per cent of the peanut meal.

Subtracting the arachin nitrogen from the total nitrogen in the extract gave a value corresponding to 9.4 per cent of conarachin.

ESTIMATION OF ARACHIN AND CONARACHIN BY MEANS OF THE REFRACTOMETER

These determinations were based on the method described by Robertson (*7*), who showed that the change in refractive indices of aqueous solutions owing to the introduction of protein is proportional to the concentration of the protein, and conforms to the formula

$$n - n' = ac$$

where n is the refractive index of the protein solution, n' that of the solvent, c the percentage of protein in the solution, and a a constant characteristic for the protein. Reiss (*5*) had previously shown that the refractive indices of closely related globulins did not differ sufficiently to distinguish between them. In the determinations reported here it was accordingly assumed that the difference in the refractive indices of arachin and conarachin was so small as to have no material significance in the results.

The measurements were made with a Carl Zeiss refractometer, and a Palo light was used. The 10 per cent sodium chloride extract of the meal used was found to have a refractive index of 1.3530, and that of the pure salt solution, 1.3510. The extract was heated carefully to 85° C., and the coagulated conarachin settled by centrifugalization. The refractive index of the clear supernatant liquid was found to be 1.3525. The percentages of arachin and conarachin were calculated as follows:

$1.3530 - 1.3510 = 0.0020$, refraction due to arachin and conarachin
$1.3525 - 1.3510 = 0.0015$, refraction due to arachin
0.0005, refraction due to conarachin

Substituting these values in the formula

$$n - n' = ac. \quad (a = 0.00236)$$

$\frac{0.00150}{0.00236} = 0.635$ per cent, or 0.635 gm. arachin in 100 c. c. of extract.

$\frac{0.0005}{0.00236} = 0.2118$ per cent, or 0.2118 gm. conarachin in 100 c. c. of extract.

Two liters, or the total extract from 50 gm. of meal, would, therefore, contain 12.70 gm. of arachin and 4.236 gm. of conarachin, or 25.4 per cent and 8.4 per cent, respectively, of the meal.

ESTIMATION OF CONARACHIN BY PRECIPITATION WITH TRICHLORACETIC ACID

One hundred cubic centimeters of the extract was made 40 per cent saturated with ammonium sulphate, and the precipitated arachin removed by filtration. Conarachin was precipitated from the filtrate by addition of trichloracetic acid. The precipitate was filtered off and washed repeatedly with small portions of dilute trichloracetic acid until all the ammonium salts were removed. The precipitate of conarachin contained 0.03933 gm. of nitrogen. The original 2 liters of extract from 50 gm. of meal would, therefore, contain 0.7665 gm. of nitrogen, equivalent to 8.4 per cent of conarachin in the oil-free meal.

The results of the various determinations of arachin and conarachin are summarized in Table 6. The percentages obtained by the various methods are in fairly close agreement.

TABLE 6.—*Percentages of arachin and conarachin in oil-free peanut meal as determined by various methods*

Method of estimation	Arachin	Conarachin
	Per cent	*Per cent*
Isolation by fractional precipitation with ammonium sulphate [a]	22.8	
	22.0	
	24.0	
Coagulation of conarachin and estimation of arachin in the filtrate	25.0	9.4
Determination by means of the refractometer	25.8	8.4
Precipitation with trichloracetic acid		8.4
Average	23.8	8.7

[a] The figures obtained by this method are somewhat too low on account of losses incident to the isolation and purification of the arachin. The quantity of arachin in the meal is nearer 25 per cent than 23.8 per cent, the average of all the determinations.

EXAMINATION OF PEANUT MEAL FOR PROLAMIN

Fifty grams of the meal was stirred for four hours with approximately 1 liter of 65 per cent alcohol. Only 1.5 per cent of the total nitrogen in the meal was extracted. The filtered alcoholic extract was evaporated to dryness. The residue consisted chiefly of a hard, yellow material which had a sweet taste, and gave a strong carbohydrate test with Molisch reagent. It contained 0.5 per cent nitrogen. From these results it was concluded that peanuts contain but a negligible quantity, if any, of the protein of the prolamin class.

EXAMINATION FOR GLUTELIN

A quantity of the peanut meal was exhaustively extracted with 10 per cent sodium chloride solution to remove the globulins, and the residue was then stirred for eight hours with 0.2 per cent sodium hydroxide solution. The alkali extracted 4 per cent of the total nitrogen of the meal. On acidification with acetic acid the extract yielded a brown substance which contained 9.52 per cent nitrogen. The character and properties of this material were not characteristic of a glutelin. This portion represents 11 to 12 per cent of the total nitrogen of the meal.

SUMMARY

Oil-free meal obtained by ether extraction of finely ground shelled Virginia peanuts was found to contain 7.36 per cent nitrogen, equivalent to 40.48 per cent crude protein (N×5.5).

Ten per cent sodium chloride solution extracted from the oil-free meal 6.41 per cent nitrogen, or 35.27 per cent crude protein.

Examination of the peanut meal showed the presence of only two globulins, arachin and conarachin, present to the extent of approximately 25 per cent and 8 per cent, respectively, of the oil-free meal.

Arachin in 10 per cent sodium chloride solution does not coagulate even at boiling temperature of the saline solution. It has the specific rotation, $[\alpha]_D^{20} = -39.5$, and precipitates completely from its sodium chloride solution at 40 per cent saturation with ammonium sulphate.

Conarachin coagulates at 80° C. It has a specific rotation, $[\alpha]_D^{20} = -42.7°$, and is precipitated at 85 per cent saturation with ammonium sulphate.

Arachin in 10 per cent sodium chloride solution was practically completely precipitated by trichloracetic, tannic, or tungstic acids. Amino acids, if present, did not come down with the arachin. In the presence of peptone trichloracetic acid precipitated all of the arachin but none of the peptone; tannic acid precipitated 39.13, and tungstic acid 44.66 per cent of the peptone nitrogen in addition to the arachin when both the latter were present in 10 per cent sodium chloride solution.

No evidence was obtained showing the presence in peanuts of significant quantities of albumin, prolamin, or glutelin.

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